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14. ABSTRACT
The overarching goal of this program is to explore our recent clinically relevant observations of DEARE in a unique cohort of non-human primates (NHP), termed the Radiation Survivor Cohort (RSC). This work consists of 4 projects: 1. Studies of type 2 diabetes mellitus
(T2DM); 2. Radiation-induced heart disease (RIHD); 3. Chronic immune impairment with restriction of the antigenic response repertoire; and 4. Genomic and transcriptomic studies of the molecular pathogenesis of T2DM, RIHD, and immune impairment in the context of DEARE.

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The **subject** of this research is the phenomenon of Delayed Effects of Acute Radiation Exposure (DEARE). The **purpose** and overarching goal of this program is to explore our recent clinically relevant observations of DEARE in a unique cohort of non-human primates (NHP), termed the Radiation Survivor Cohort (RSC), and a smaller more controlled group of NHP exposed to radiation prospectively, termed the Prospective Radiation Cohort (PRC). The **scope** of this work work consists of 4 projects making use of these unique NHP resources: 1. Studies of type 2 diabetes mellitus (T2DM); 2. Radiation-induced heart disease (RIHD); 3. Chronic immune impairment with restriction of the antigenic response repertoire; and 4. Genomic and transcriptomic studies which will inform us regarding the molecular pathogenesis of T2DM, RIHD, and immune impairment in the context of DEARE. Work during the first year of this program has proceeded as planned, with slight logistical modifications but no major changes or delays.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Radiation; delayed effects; late effects; heart; immune system; type 2 diabetes mellitus; genomics; transcriptomics; *Mus musculus*; *Macaca mulatta*.

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

(See next page)

Major goals are listed below by project, including relevant dates and percentage completion as indicated.

Core Primate Studies (Cline)

SA1: Provide NHP Investigative Platform

Major Task 1: Regulatory Approval (months 1-6)
Milestone: Local Institutional Animal Care and Use Committee (IACUC) and DOD Animal Care and Use Review Office (ACURO) approvals in place (100%)

Major Task 2: Access to Long Term Radiation Survivor Cohort (months 1-6)
Milestone: Scheduling and meetings in progress (75%)
Sample collections have begun, in support of all projects (planned for months 6-60; 20% complete)

Major Task 3: Acquisition of Prospective Cohort (months 1-6)
Milestone: Animals arrived at Wake Forest 2/9/16 (100% completed).

Major Task 4: Irradiation and Study of Prospective Cohort
Milestone: Irradiations planned for quarter 4 (100% completed in Y2Q1)

SA2: Promote Synergy and Productivity

Major Task 5: Data management/tracking, group interactions (months 1-6)
Milestones: Data management (100% completed for year 1)
Establish monthly web meetings (100% complete)
Annual strategic retreat to be scheduled (100% complete for year 1)
Publications (months 6-60; 10% complete in the context of 5 year plan, first cardiac paper submitted and accepted; abstracts accepted for the 2016 Radiation Research Society Meeting)

SA3: Facilitate Translation and Therapeutic/Preventive approaches

Major Task 6: Strategize towards clinical implementation and future funding
Milestones: Promote new grant submissions (months 36-60; 0% complete)
Progress update at end of year 2 (0% complete)

Project 1: Diabetes (Kavanagh)

SA1: Document the time course of IR-mediated reduced insulin sensitivity

Major Task 1: PRC - Prospective Cohort
Milestones: Create a longitudinal clinical profile of diabetes mellitus (DM) risk

with irradiation (15% achieved in context of 5 year plan)
Local IACUC approval (100% achieved)
ACURO Approval (100% Achieved)

Major Task 2: RSC - Radiation Survivor Cohort
Milestones: Generate comparable clinical data in monkeys with DM as DEARE to compare with the earlier prospectively irradiated monkeys (60% achieved in context of 5 year plan)

SA2: Examine IR-related increases in oxidation of tissue proteins over time

Major Task 3: Redox Proteomics of prospective and long-term irradiated tissue
Milestones: Compare and contrast early and late changes in redox proteomics following irradiation in mice and monkeys. Verify the role of Akt protein in DM. Identify individual or protein clusters with long-term modifications following irradiation. (25% achieved in context of 5 year plan)

SA3: Track extracellular matrix (ECM) remodeling, including fibrosis, in muscle and adipose depots after irradiation

Major Task 4: ECM characterization and magnetic resonance imaging (MRI).
Milestones: Characterize tissue changes in both prospective and long-term IR monkeys and relate these to in vivo insulin sensitivity assessments. Validate the utility of MR imaging to detect qualitative tissue changes (20% achieved in context of 5 year plan)

SA4: Evaluate therapeutic agents that reduce fibrosis and restore fat expansion to mitigate metabolic changes with IR.

Major Task 5: Mouse intervention study
Milestones: Prevent metabolic disease development in IR mice with interventions. Demonstrate reductions in protein modifications and/or tissue remodeling with treatment (8% achieved in context of 5 year plan).

Project 2 – Radiation-Induced Heart Disease (Register)

We proposed 3 inter-related Specific Aims across the 5 Year Grant Period.

SA 1: Determine the Effects of Total Body Irradiation on NHP Cardiac Structure and Function.

Major Task 1: Cardiac Imaging and Sample Collection

Novel cardiac MRI approaches will be used to evaluate myocardial tissue fibrosis, a key component of RIHD, in order to follow changes in myocardial phenotypes in animals across time. Additional structural and functional cardiac phenotypes to be assessed by echocardiography/MRI include left ventricular (LV) mass, posterior and interventricular wall thickness, right ventricular thickness, and aortic wall thickness. Functional parameters will include systolic and diastolic function, ejection fraction, aortic stiffness/pulse wave velocity, wall motion, and electrocardiography. Grid tag mapping will be also used to assess strain and LV volume.
Scheduled Dates: Months 6-48
Milestones: Percent completion - 10% in context of 5 year plan (echocardiographic measurements taken from the long term and prospective cohorts; echo data obtained for the prospective cohort, established MR protocols to assess extracellular matrix volumes as well as cardiac perfusion along with parameters noted above, baseline cardiac MRI scans for the prospective cohort were completed in August/Sept 2016).

SA 2: Identify Novel Molecular Biomarkers of Cardiovascular Injury and RIHD

Major Task 2: Biomarker Analysis - Analyze established and emerging serum markers of cardiac damage implicated in cardiomyocyte death (microRNAs such as miR133a) and extracellular matrix remodeling (type I and III collagen propeptides and degradation products). Circulating miRNA levels and serum extracellular matrix turnover biomarkers will be evaluated in relation to structural and functional phenotypes and histologic and biomolecular features of myocardial tissues and differentially expressed myocardial mRNA and miRNA which will be identified through expression profiling of heart tissues obtained at necropsy.
Scheduled Dates: Months 6-48
Milestones: Percent completion - 0% (preparation under way; permissions and procedures for analysis)

SA 3: Determine the Efficacy of Candidate Mitigating Agents on RIHD.

Major Task 3: Assessment of Mitigating Agents. We proposed to retrospectively and prospectively assess cardiac and coronary artery injury in NHP previously exposed to mitigating agents, including the long-term RSC cohort and past studies of growth factors (e.g. HGH), superoxide dismutase mimetics, and novel therapeutics. We will use the pathologic and biomarker approaches noted above, including in vivo assessments. These results will be critical to our understanding of RIHD. The preliminary data suggests that the changes in the heart based on the radiation dose are significantly greater that would have been predicted from prior murine studies.
Scheduled Dates: Months 6-48
Milestones: Percent completion - 10% complete in context of 5 year plan (assessed effects of a mitigating agent (superoxide dismutase analogue, hexyl MnSOD) on myocardial gene expression, collected cardiac tissues from Project 1 rodent study assessing the effects of a heat shock protein inducer on insulin sensitivity, other

preparations under way**)**

Project 3 – Immune Recovery (Duke Consortium – Chen & Sempowski)

SA 1: Define the roles of thymopoiesis and peripheral expansion in overall T cell reconstitution after radiation-induced injury based on radiation dose.

Major Task 1: NHP Studies (5% complete in context of 5 year plan, first analysis in progress)
Milestones:

1.1. Phenotypic analyses in long-term cohort (60 months)
1.2. sjTREC in long-term cohort (60 months)
1.3. Phenotypic analyses in prospective cohort (60 months)
1.4. sjTREC in prospective cohort (60 months)
1.5. Determine primary immune response to influenza vaccine (36 months)
1.6. Determine primary and recall immune response to influenza vaccines (60 months)

Major Task 2: Murine Studies
Milestones:

2.1. Local IACUC Approval (3 months, 100% complete)
2.2. Determine the roles of thymopoiesis and peripheral expansion in overall T cell recovery in the classic radiation injury model (18 months, 40% complete in context of 5 year plan)
2.3. Determine the roles of thymopoiesis and peripheral expansion in overall T cell recovery in the second radiation injury model (24 months, 0% complete)

SA 2: Determine if therapeutic agents identified in our previous studies are able to promote or accelerate overall T cell immunity after radiation injury.

Major Task 3: HGH Mouse Studies
Milestones:

3.1. Determine the effect of HGH on phenotypic T cell reconstitution (36 months, 0% complete)
3.2. Determine the effect of HGH on functional T cell recovery (48 months, 0% complete)
3.3. Determine the mechanism by which HGH promote T cell reconstitution (60 months, 0% complete)

Major Task 4: IGF-1 Mouse Studies
Milestones:

4.1. Determine the effect of IGF-1 on phenotypic T cell reconstitution (36 months, 0% complete)
4.2. Determine the effect of IGF-1 on functional T cell recovery (48 months, 0% complete)

4.3. Determine the mechanism by which IGF-1 promote T cell reconstitution (60 months, 0% complete)

Project 4 - Genomic Sequencing and Stem Cell Lines (Duke Consortium – Dave)

Aim1: Define the genetic mutations and gene expression signatures induced by DEARE

Milestones: DNA Sample extraction (months 1-36) - 10% complete in context of 5 year plan; in methods development
Exome analysis (months 13-60) - 10% complete/5 years
RNAseq (months 6-48) - 0% complete
RANseq analysis (months 12-60) - 0% complete

Aim 2: Determine the molecular basis of lymphoid immunodeficiency induced by DEARE.

Milestones: Generate/characterize B cell lineages (months 30-60) - 5% complete/5 year plan
Generate/characterize T cell lineages (months 30-60) - 5% complete/5 year plan

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Core Primate Studies (Cline)

1) Major activities:

Protocol approvals for all animal studies; establishment of subcontracts and accounts; development of a meeting schedule; review of goals and plans with all investigators; hiring/allocation of effort for personnel; acquisition of NHP subjects for the prospective cohort; pre-irradation physical examinations, imaging, and blood sampling for all projects; total body irradiation.

Characteristics of our animal populations are briefly described below.

2) Specific objectives:

As per SOW. Regulatory approvals, establishment of meeting schedule, hiring, planning, animal acquisition, irradiation.

3) Results/key outcomes:

- All animal protocol approvals complete at local and ACURO level
- Subcontracts and accounts established
- Goals and plans reviewed with all investigators
- Monthly web conferences established
- Hiring/effort allocations made as planned
- Secure server folders established for data exchange
- NHP subjects acquired for PRC
- Sampling and data acquisition on schedule
- Slight delay in irradiation date (Sept 2016 Y1Q4 to Oct 2016 Y2Q1)
-

4) Other achievements:

Data presented at the annual national meeting of the Centers for Medical Countermeasures against Radiation and at local Wake Forest and Duke venues.

A brief overview and synopsis of major findings is presented below.

<u>The Radiation Survivor Cohort (RSC)</u>

The population of the RSC is shown graphically in Figure 1 (see next page). Briefly, this cohort consists of rhesus macaques (*Macaca mulatta*) irradiated as juveniles or adults, in the range of 3-8.5 Gy via single total body exposure, at a dose delivery of 60-80 cGy/min. The cohort is supported by the National Institute of Allergy and Infectious Disease (NIAID), which is leveraged by this DOD Focused Program award. Animals were acquired between 2007 and 2016 through an “adoption” strategy from multiple sites. The RSC currently consists of 85 irradiated animals and 15 controls. The population has a 5:1 male:female ratio. Animals are housed socially, primarily in pens, and fed a custom-formulated “Typical American Diet”. All animals receive daily environmental enrichment and are part of a behavioral management program. Approximately one-third of the animals were given mitigating agents (e.g. antibiotics

or growth factors) during the acute period of hematopoietic injury. This cohort is studied using a multidisciplinary approach to long-term morbidity/mortality. Notably, some animals are now 10 years out from irradiation, providing a unique view of DEARE.

Animals in the RSC undergo a regular schedule of medical care and assessment, including but not limited to:

Daily
- Twice-daily clinical observations
- Environmental enrichment

Biweekly
- Cognitive testing (subset)

Monthly
- Physical exam
- Blood collection for experimental measures and archive
- Urine collection for experimental measures and archive
- Body weight

Semiannually
- Complete blood counts
- Clinical chemistry panels
- Testing for diabetes mellitus (hemoglobin A1c, glucose, with glucose tolerance testing and mimimal model testing on a subset)
- Anthropometrics (trunk length, waist diameter)

Annually
- Flow cytometry (blood, see Project 3)
- Bone marrow aspirate
- Echocardiography
- Computed tomography scan (whole body)
- Renal ultrasound
- Gastrointestinal endoscopy and biopsy
- Bronchoalveolar lavage
- Cranial MRI (subset, 3 year rotation)
- Ocular exam

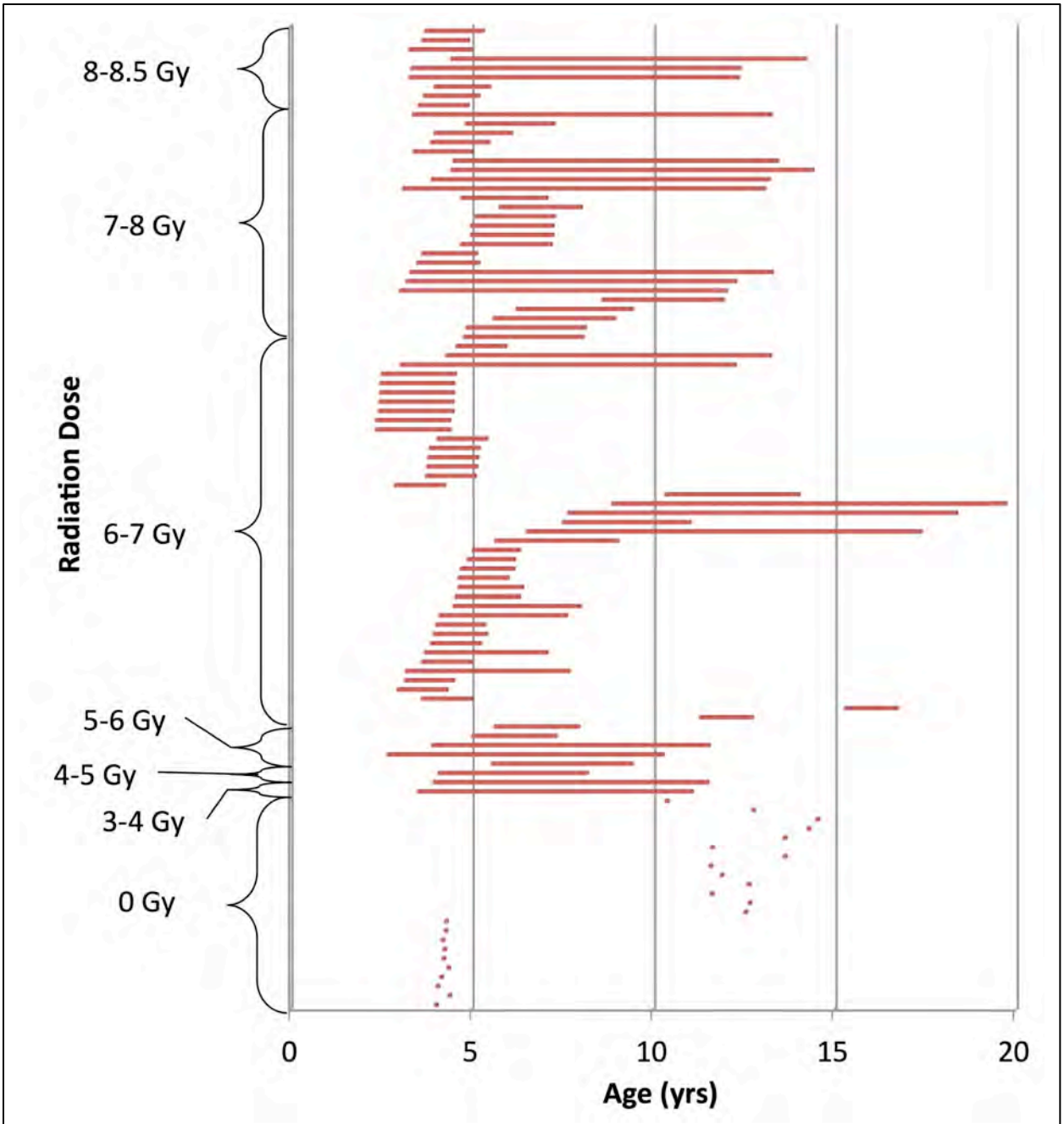


Figure 0.1: Age and dose distribution of the RSC. Each horizontal bar represents a single animal; the left end of the bar is the age at which the animal was irradiated, and the right end of the bar is the animal's current age or age at time of death.

All animals are given routine veterinary care, and we have established clinical criteria for identification and management of animals with health abnormalities. Major patterns of morbidity in the RSC are summarized in Table 1.

A variety of morbidities have been identified in the RSC; detailed quantitative and statistical analyses are in progress. Incidence data are shown below.

Table 0.1: Threshhold-based determinations of morbidity in the long-term Radiation Survivor Cohort.

Organ/Disease	Irradiated	Control	Criteria
Diabetes	7.2% (6/83)	0% (0/15)	HbA1c >6.5 or 3 Fasting Blood Glucose > 100 mg/dL or Any Non Fasted Blood Glucose > 200 mg/dL
Kidney	6% (5/83)	0% (0/15)	Bun > 30 mg/dl Cr > 1.1 mg/dl Loss of renal volume >50%; Stones
Lung	10.8% (9/83)	13.3% (2/15)	CT densities (any); bullous emphysema >25% of lung volume Hypoxia under sedation (SPO2 < 80% or requiring oxygenation)
GI	6% (5/83)	6.7% (1/15)	Any lesion on endoscopy Chronic diarrhea (severity code >2 for >5 days)
Skin	22.9% (19/83)	6.7% (1/15)	Dermatitis or other significant disease
Heart/CV	25.3% (21/83)	20% (3/15)	Murmur/valvular insufficiency on echo; MAP >120 Stroke volume <5 mls/stroke & Cardiac output <0.5 L/min at >7 y
Brain (any lesion)	13.3% (11/83)	0% (0/15)	Y/N MRI lesions visible on SWI Neurologic abnormality on clinical exam
Eyes (cataracts)	21.7% (18/83)	0% (0/15)	Y/N
Behavior	9.6% (8/83)	13.3% (2/15)	Requiring management (customized enrichment plan or medication)
Neoplasia	2.4% (2/83)	0% (0/15)	Y/N
Testicular atrophy	21.2% (7/33)	0% (0/15)	<10 ml testis volume after 7 years of age; Vol = (p/6)(LxW^2)
Ovarian dysfunction	Pending	Pending	No menstruation; LH > 30 ng/ml Or FSH > 3 ng/ml
Bone	34.1% (15/44)	0% (0/13)	BMC < 274g BMD < 0.373 g/cm^3 (- 2 SDs) (Excluding animals <7 yrs old). CT: Fracture or other gross abnormality
Obese **Underweight**	7.2% (6/83) 22.9% (19/83)	53.3% (8/15) 0% (0/15)	High: Waist Circumference > 45 cm or Dexa Body Fat > 30% Low: Waist Circumference < 25 cm or Dexa Body Fat < 12.3% (Excluding animals under 7 yrs old)

The Prospective Radiation Cohort (PRC)
The study design for the PRC is shown below. These specific-pathogen-free animals were acquired and quarantined in year 1 and irradiated in October 2016. Imaging and sample collections are proceeding as per the program statement of work and the needs of the individual projects.

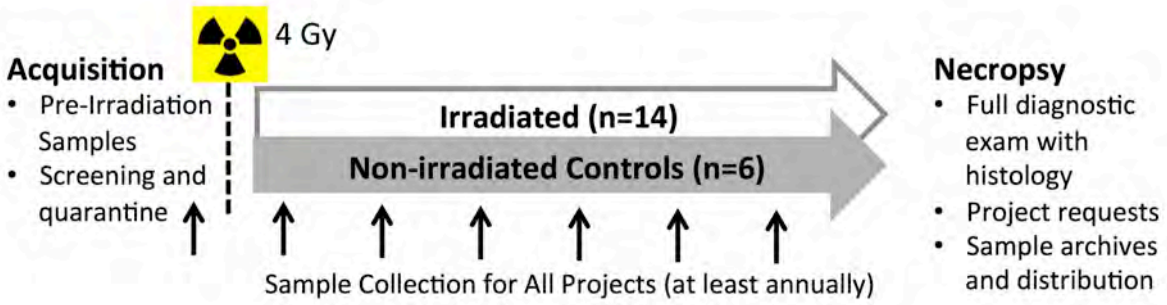


Figure 0.2: Study design for the Prospective Radiation Cohort (PRC).

Project 1 – Diabetes (Kavanagh)

1) Major activities

Radiation Survivor Cohort
Procedures have included analysis of CT scans for body fat distribution; glucose tolerance testing including homeostatic modeling; and biopsy-based testing of skeletal muscle signaling and composition.

Prospective Cohort
Major activities included collecting baseline samples and testing of monkeys prior to irradiation including CT scans, blood sampling, glucose tolerance, and biopsy.

Mouse Studies
Major activities include a novel study of dietary DM induction in mice and establishment of a platform to induce tissue fibrosis and insulin resistance. In subsequent years we will initiate therapies to reduce fibrosis in musculature and evaluate subsequent effects on glucose metabolism in high fat fed mice.

2) Specific objectives

Specific objectives for this period included analysis of RSC data to characterize radiation-associated DM; Stratification of the PRC monkeys to ensure comparability of A1c and bodyweights across groups; validation of the mouse radiation-induced DM model; and optimization of procedures to be used in future years.

3) Results/key outcomes

Radiation Survivor Cohort

The RSC animals have a number of unique features that accompanying their DM presentation. The first was that obesity is seen in the absence of general obesity and loss of visceral fat. Data is shown in the graphs below with all individuals matched by age and representing non-irradiated control monkeys (Non-Rad CTL), monkeys from the RSC that were non-diabetic (Rad CTL) and monkeys from the RSC that had DM (Rad DM). There was a radiation effect ($p<0.05$) on visceral fat, such that they had less which is in conflict with the general observation that metabolic disease develops with the accumulation of ectopic fat, particularly in the abdominal compartment. In all graphs shown, differences between groups of monkeys are indicated by unlike letters.

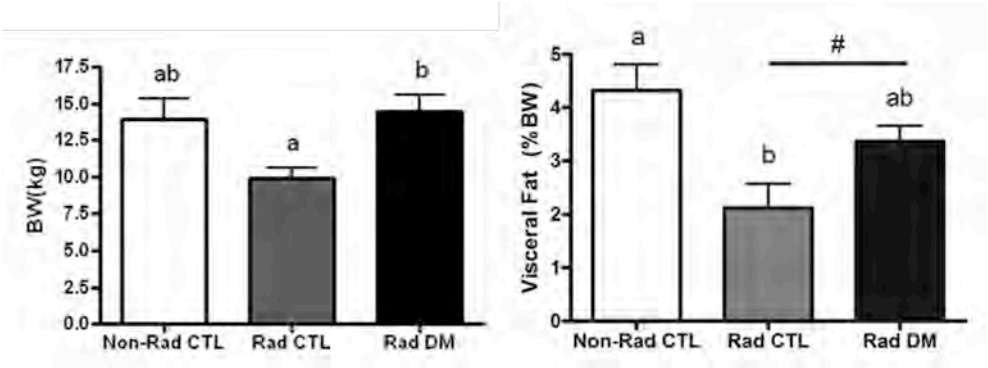


Figure 1.1: Irradiated monkeys developing diabetes (DM) years after exposure were non-obese and radiation actually decreases visceral fat. This is not typical for type 2 DM.

We further evaluated their ability to metabolize glucose. The DM monkeys had insulin resistance and hyperglycemia, as indicated by elevated insulin resistance scores (HOMA), A1c and glucose clearance following glucose or meal challenge (GTT and MTT respectively).

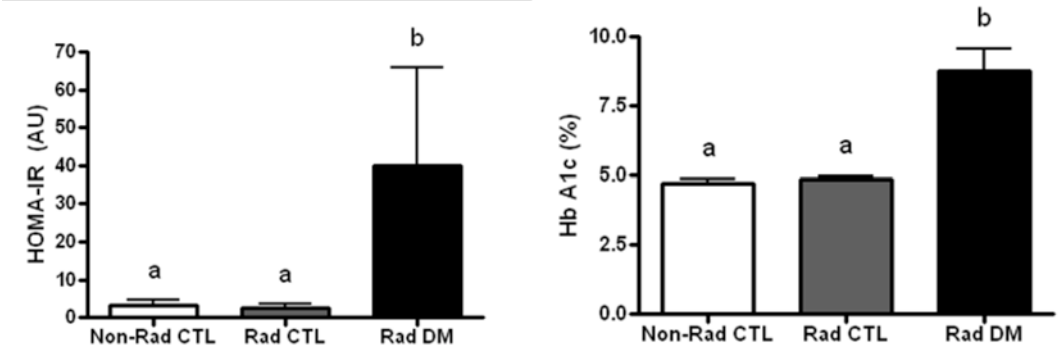


Figure 1.2: DM developing after radiation exposure is typical of type 2 with insulin resistance (elevated HOMA scores) and time-averaged hyperglycemia (HbA1c %)

From the glucose tolerance testing, we noted that pancreas function looked normal (acute insulin release post-glucose was within 10% of both non-DM monkey groups ANOVA $p=0.77$).

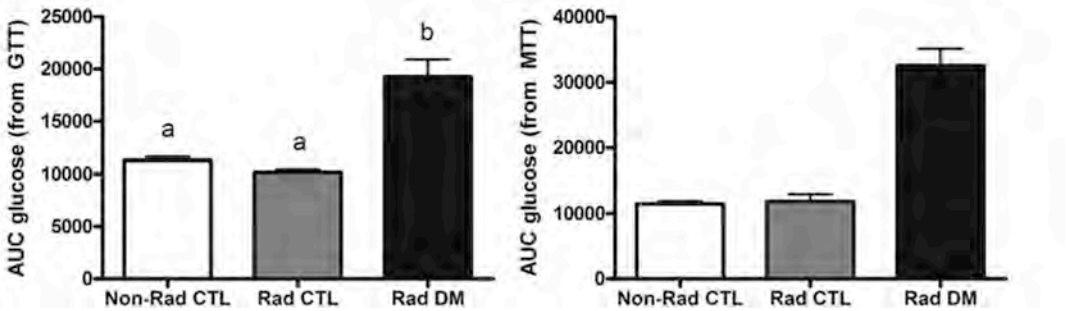


Figure 1.3: DM monkeys are unable to metabolize glucose challenges following intravenous dextrose (GTT) or oral meal (MTT) tolerance testing. Non-DM monkeys that have been irradiated had normal capacity for glucose disposal.

The bulk of glucose is metabolized by muscle tissue, so we evaluated the effect of insulin on muscle and found that radiation exposure decreases the ability of muscle to respond normally. The graphs below show that proteins in the insulin signaling pathway (IRS-1 and Akt) were inadequately activated in DM with even non-DM irradiated monkeys having deficiency.

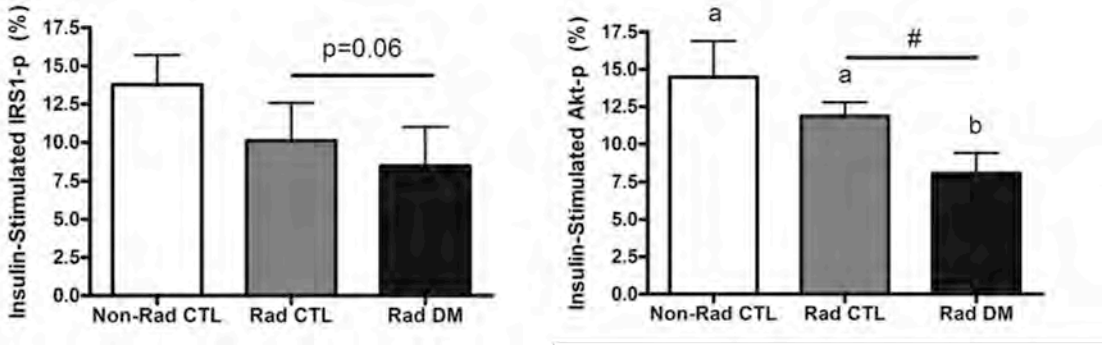


Figure 1.4: Radiation exposure reduces the ability of insulin to act on muscle tissue. DM monkeys are most severely affected but even non-DM radiation exposed monkeys had deficient insulin response.

We then hypothesized that radiation-induced insulin resistance may result of increased fibrosis in the muscle. Fibrosis is a well-known consequence of radiation damage and accumulation of collagens in muscle can interfere with diffusion of insulin from the capillary to the muscle cell, and the growth and remodeling of capillaries within the muscle tissue. In the graphs below, radiation has a significant effect on increasing relative levels of collagen type 4, which is associated with the basement membranes of cells and could interfere with insulin access and function as described. This radiation-related increase in collagen 4 is supported by a trend for increased tissue growth factor beta (TGFß) with radiation as this is the primary signaling for collagen production in cells.

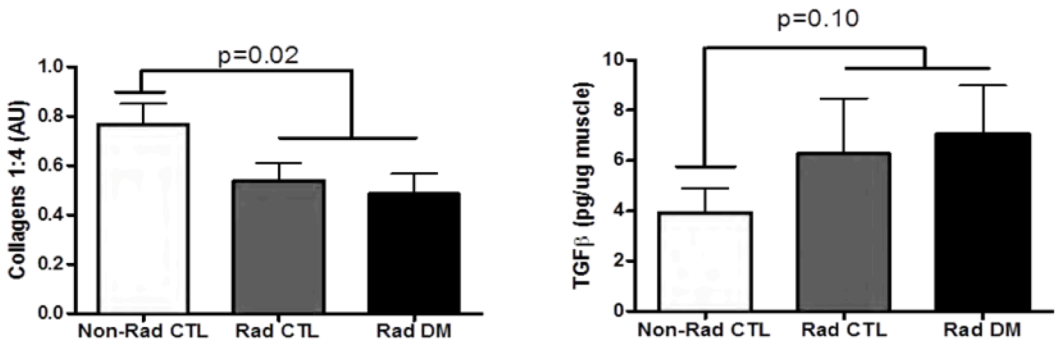


Figure 1.5: Radiation exposure leads to greater pro-fibrotic signaling (TGFß) and deposition of the extracellular matrix component, collagen 4.

Collagen 4 accumulates because of increased TGFß signaling in the absence of increased breakdown. We measured matrix metalloproteinase activity (MMP) which degrades extracellular matrix proteins such as collagen 4 and levels were not different (p=0.39).

We have continued to investigate why some individuals develop DM as a delayed effect of radiation despite similar pro-fibrotic changes in muscle. As noted earlier, muscle is responsible for 90% of glucose disposal out of the circulation and thus is circulatory capacity deficient in muscle, this will further reduce glucose use by muscle. We measured the number of capillaries (by counting endothelial cells which are marked as $CD31^{+}$), and the circulatory signal for vasodilation (nitric oxide metabolite NO_3). These biomarkers of muscle perfusion suggest that DM can be prevented post-radiation by enhanced blood flow in muscle.

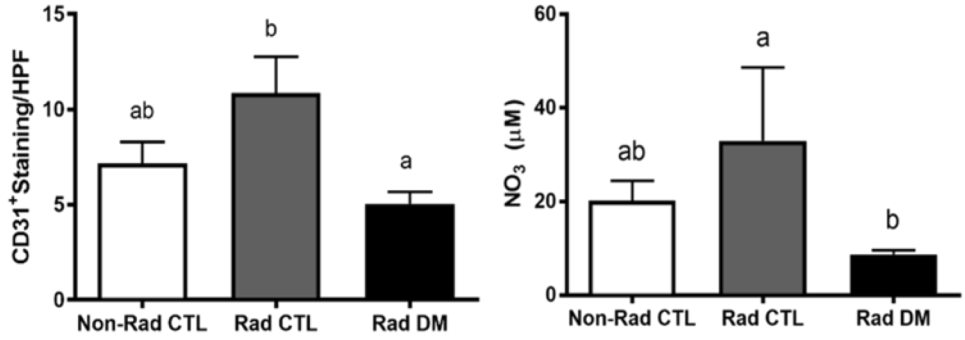


Figure 1.6: Muscle endothelial cell numbers (CD31+ cells) and the biomarker of vasodilatory product nitric oxide (NO3) suggest that DM results from deficiency, and that maintaining normal glucose disposal (Fig 2+3) is related to maintaining high normal indicators of muscle blood perfusion.

Capillarization is known to be important in muscle function and insulin function in both normal and older humans. This process is mediated in part by transcription factors

VEGF and TNFR1A, which shown in the table below were not different between groups. Nor was endothelial nitric oxide synthase (eNOS) gene expression in muscle different despite its product, NO_3, showing group differences (above).

Table 1.2: No differences in vascular growth signaling (VEGF or TNFR1A) or gene expression of endothelial nitric oxide synthase (eNOS), which produces NO3 (Fig 6) was seen in muscle.

	Non-Rad CTL	Rad CTL	Rad DM	Overall p-value	Radiation effect p-value	Diabetic effect p-value
VEGF (pg/ug muscle protein)	11.45(2.11)	10.05 (1.18)	11.49 (1.52)	0.78	0.74	0.71
TNFR1A (pg/ug muscle protein)	7.39 (0.98)	13.2 (3.68)	7.85 (2.91)	0.13	0.34	0.53
ln eNOS (AU)	1.012 (0.28)	1.069 (0.30)	1.634 (0.69)	0.90	0.93	0.67

We have assessed an addition 24 animals for the endpoints shown in the graphs and tables above to increase power and evaluate monkeys that are metabolically abnormal but not yet DM. These animals are hoped to fill the data gap between the gray and black bars on our graphs, and strengthen our hypothesis that skeletal muscle perfusion needs to be enhanced to prevent DM development post-irradiation.

<u>Prospective Radiation Cohort</u>

As noted above, we have brought in 20 new younger animals to be irradiated and get data that tracks the early progression of these changes in fibrosis and perfusion. As mentioned, 4 years was the shortest period post-irradiation that we have available in the RSC. These new animals, referred to as the Prospective Radiation Cohort (PRC), will have baseline and 0-2 year data collected to fill that gap. These animals were stratified to radiation or control conditions to ensure that groups were comparable on weight and A1c measures as shown below. There were no statistically significant differences between these variables prior to irradiation.

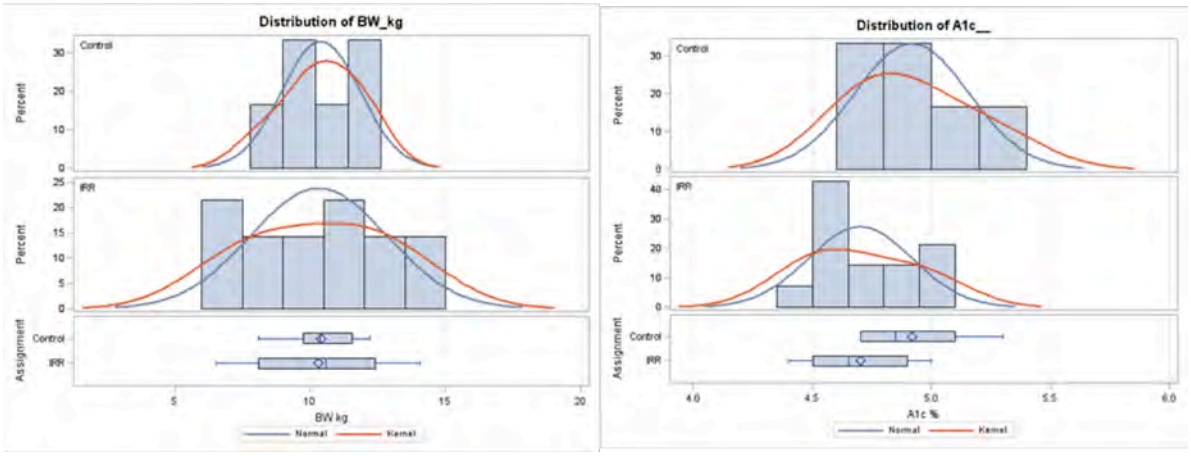


Figure 1.7: New monkeys that will have baseline assessments prior to radiation exposure and follow up have been stratified to control and radiation conditions, with

their bodyweight and glucose control equivalent between groups.

We have developed proteomics methods with Dr. Cristina Furdui for measurement of modified skeletal muscle proteins. Our hypothesized target is Akt (as shown to be deficient in activity above) and with this methodology we will be able to identify specific redox changes in isoforms Akt1 and 2. Protocols for muscle tissue have been developed and 40 monkey muscle samples have been delivered to the Furdui laboratory for analysis. This analysis is major activity planned for the second year.

Mouse Studies

Our specific aim 4 is an interventional DM prevention trial in irradiated mice. In this aim we will initiate therapies to reduce fibrosis in musculature and evaluate subsequent effects on glucose metabolism in high fat fed mice. The diet exposure is similar to that being fed in the monkey studies. To ensure that we have a good model to test these outcomes, we have completed a trial administering 7 Gy to the whole body, administered as 2 doses administered 10 minutes apart. Mice of both sexes were acclimated to the high fat diet prior to irradiation and we have done assessments at 1 and 3 months following exposure (<10% of lifespan). The graph below shows that after a brief dip in weight associated with acute radiation sickness, that recovery and equivalent weight is achieved. All mice were equivalently exposed to antibiotics.

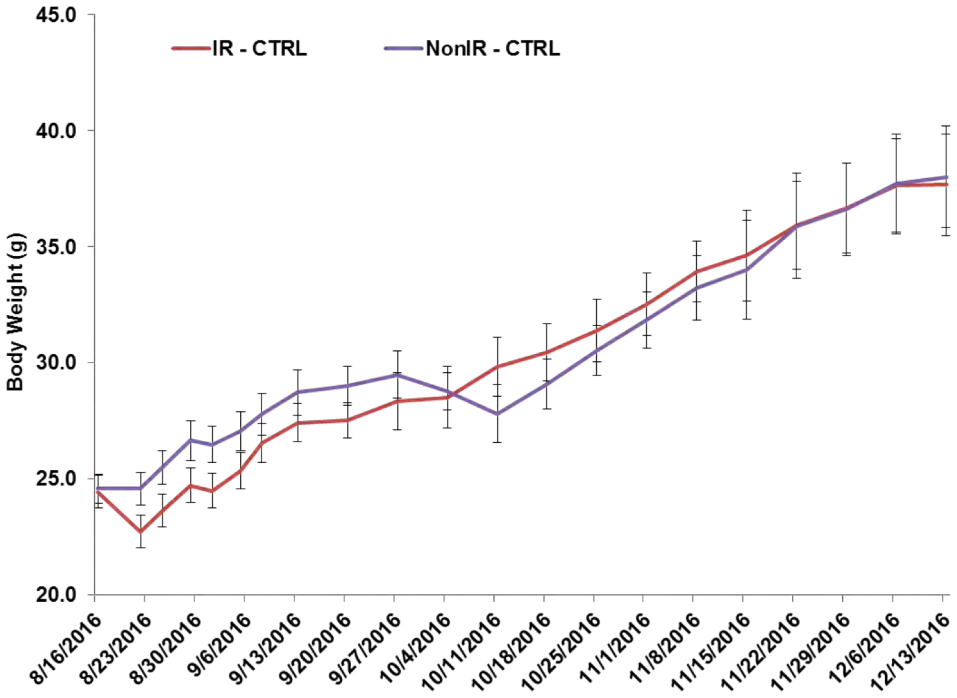


Figure 1.8: Mice exposed to 7 Gy whole body radiation recover and have comparable bodyweights to non-irradiated mice. All mice are consuming a typical Western diet.

Glucose tolerance testing at 1 month shows that total body irradiation (red bar) results in greater area under the curve as compared to control mice (purple). Insulin

tolerance testing at 3 months confirms insulin resistance and elevated fasting glucose values.

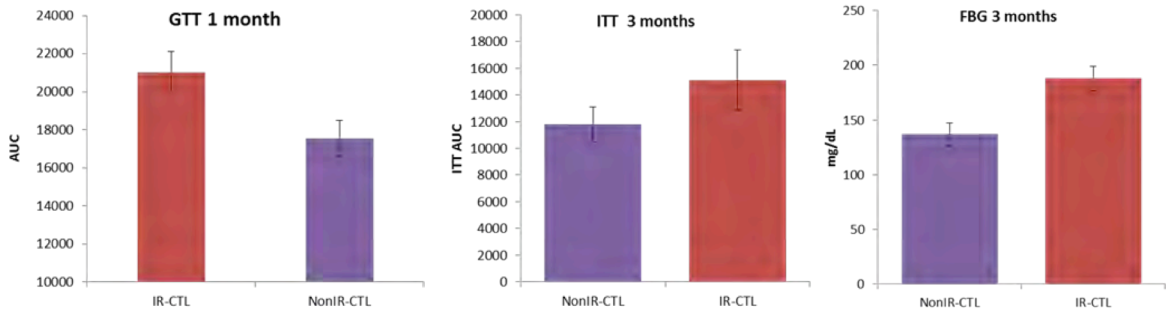


Figure 1.9: Mice exposed to 7Gy whole body irradiation develop insulin resistance and hyperglycemia, and thus are a good model for testing interventions.

These results give us a strong foundation for the studies that may be effective in preventing DM after radiation exposure. A model of DM post-irradiation in rodents has not yet been fully described in the literature and thus the publication of these data will fill this gap and substantiate DM as a delayed effect of radiation. Our interventional mouse studies are planned to begin in January 2017 (Year 2 of the project).

4) Other achievements:

We have piloted methods that will be used in Aim 3 mouse studies and made refinements to ensure this study will be successful. We have generated protocols for Aim 2 proteomic analysis of muscle tissue from mice and monkeys.
We have initiated collaborations for microdialysis methods that will enhance measures of muscle fibrosis and perfusion in vivo.
We have also developed new collaborative efforts to measure tissue nitric oxide (NO) levels, which our preliminary data suggests will be different in diabetic and non-diabetic radiated monkeys.

Project 2 – Radiation/Heart Disease (Register)

1) Major Activities

a. Blood collections: Collection of baseline samples from the prospective cohort, and samples from the long term cohort for future biomarker analyses in progress.
b. ECM Biomarkers: Nordic Biosciences (NBS; Denmark): Validating initial candidate biomarkers of ECM turnover (titin, versican, collagen markers). Establishing Material Transfer Agreements with NBS for:
   1) transfer of ECM MAbs from NBS to WF for use in IHC studies
   2) transfer of NHP serum samples from WF to Denmark. We are working on details for CITES permits for shipping samples to NBS. We have identified key

samples from RSC and past randomized trials to include in this first shipment. Prospective Cohort samples will be shipped after collections of post radiation samples.

c. Cardiac biomarkers: Arrangements with internal Clinical labs finalized for running research samples through automated ultrasensitive clinical analyses (Siemens)
d. Cardiac tissue analyses: Consolidation of myocardial and coronary artery tissue blocks (RSC, & past Project with mitigator) for sectioning and assessment of CAA. Staining of sections for collagen content (Masson's trichrome) has been conducted. Ryne Debo has interesting gene expression from a past mitigator project which warrants pursuit of histologic outcomes.
e. Training: Image Analysis Software, Cardiac Ultrasound, and Cardiac MRI:
   - Tissues: VisioPharm, programmable, working to develop semi-automated programs to assess myocardial phenotypes including fibrosis.
   - Cardiac Ultrasound: CV ultrasound applications expert from GE provided a day long training for investigators and staff, establishing new SOPs for prospective cohort.
   - Cardiac MRI: Register and Michalson had a training session on the use of MIMP software useful for assessments of CMRI images by the developer (Craig Hamilton),
f. Ultrasound Infrastructure. Backed up all studies to local external hard drives to protect the data, and worked with WF IT to establish procedures for transfer studies to Comparative Medicine Paxcera PACs server (part of recent G20 infrastructure funds). Studies now automatically transferred to PACs.
g. Ultrasound Data: Echocardiographic images on the prospective cohort acquired by DOD team.
h. Cardiac MRI Infrastructure: Developed MR protocols with Cardiology, BME colleagues. Developed an adenosine stress perfusion protocol which was used in the cardiac MRI studies. Worked with institution to add capabilities to MR infrastructure (infusion pump and BP monitors for NHP).
i. Refined heart collection and processing protocol (SOP, Kris Michalson).
j. Baseline CT images acquired for Prospective cohort, positioning for whole body scans. CT studies were copied to PATHVMA PACs server. Review of CT scans revealed microchips in majority of the animals. Chips were surgically removed when possible, some chips remained but did not appear to significantly impact cardiac images, although there was signal interference within the immediate site of the chip.
k. Worked with Dr. Kavanagh's group (Project 1) to collect blood and tissue samples from the long term cohort. Developed protocol for assessment of adipose phenotypes using Nexcelom cellometer system.
l. Developed sampling protocols for assays and analyses for the prospective cohort, ongoing planning across projects 1,2,3,4 for coordination of assessments.
m. Conducted discussions with Duke investigators on cross-project technical collaboration on cell collections, processing, and analysis.

2) Specific objectives

Planning for new cohort animals and sampling protocols.

3) Results/key outcomes

A manuscript detailing observations of long term effects of prior irradiation on cardiac fibrosis and other phenotypes was submitted to Radiation Research Journal, received favorable reviews and is now published, and a key figure was selected for the cover of the journal. This manuscript was a joint effort of Drs. Register, Dr. Kris Michalson, Dr. Mark Cline, and others of the team. The results and key outcomes within this paper are described below.

Cardiac Structural and Functional Phenotypes: Cardiac tissues were available from fourteen irradiated and 3 control animals. Elapsed time between irradiation and death was 5.6 ± 0.59 years (range 2.2 - 9.25). Figures 2.1A-B are photomicrographs of trichrome stained sections of the LV, illustrating loss of normal cardiomyocyte architecture and replacement with collagen and fat in the radiation exposed animal. Compared to the myocardium of control animals, irradiated myocardium often exhibited separation of cardiac myofibers by intervening fibrous connective tissue, loss of normal architecture, with deranged cellular orientation and enlargement of cardiac myocytes (Figure 2.1 C-F). Gross and qualitative microscopic histological analysis of the 17 hearts available from euthanized animals revealed a significantly higher incidence of myocardial fibrosis in 9 out of 14 irradiated animals compared to none of the 3 control animals (p=0.04, Chi-square) (Table 2.1). Histomorphometric analysis of Trichrome stained sections of myocardium from these animals revealed a mean of 14.9% (± 1.35) percent collagen in irradiated compared to 9.1% (±0.86) in control animals (p=0.17, t-test).

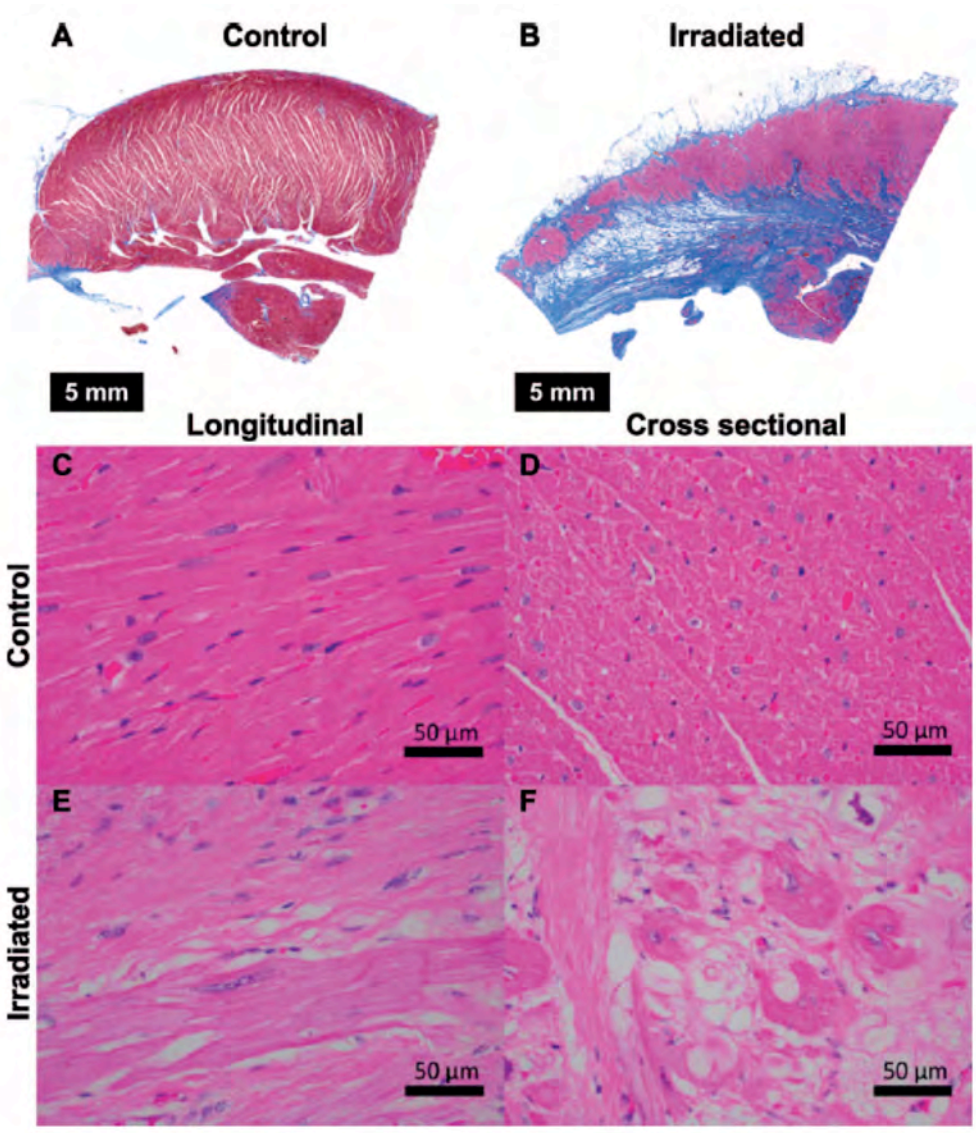


FIG. 1. Effects of prior exposure on indices of cardiac fibrosis: Panels A and B: Trichrome-stained sections of myocardium from the left ventricle of a control animal (panel A) and of a 6.5 Gy irradiated animal 2.5 years earlier (panel B) (scale bars ¼ 5 mm). Panels C–F: Longitudinal and cross-sectional H&E stained myocardium. Panels C and D: control myocardium. Panels E and F: Irradiated myocardium exhibiting marked separation of cardiac myofibers by intervening fibrous connective tissue, loss of normal architecture, cellular orientation and enlargement of cardiac myocytes.

Table 2.1: Frequency distribution of cardiac fibrosis in irradiated and non-irradiated rhesus macaques.

	No fibrosis	Fibrosis	Total
Control	3	0	3
Irradiated	5	9	14
Total	8	9	17
Odds ratio	-	-	12
P value	-	-	**0.04**[a]

[a]Significance (P>0.05) indicated in bold face.

Echocardiographic Phenotypes: Selected outcomes for echocardiographic phenotypes are shown in Figure 2.2. Thirty-one monkeys were evaluated annually for 3 consecutive years using echocardiography. The estimated mean age at the final assessment for the 11 control animals was 11.2 (± 0.3) years, compared to 11.8 (± 0.5) years for the 20 irradiated animals. At the final echocardiographic assessment monkeys had an average post-exposure interval of 8.6 ± 0.2 years and had been consuming the western diet for an average of 3.7 ± 0.1 years. Echocardiographic LVDd and LVDs were significantly smaller in irradiated animals compared to controls (LVDd: p=0.001 and LVDs: p=0.008, RMANOVA, Figure 2.2 A-B). Correction for body surface area (BSA) did not significantly alter outcomes (LVDd: p=0.007 and LVDs: p=0.017, RMANOVA, data not shown). Irradiated animals tended to have greater LVDFS% (p=0.054, RMANOVA) (Figure 2.2 C).

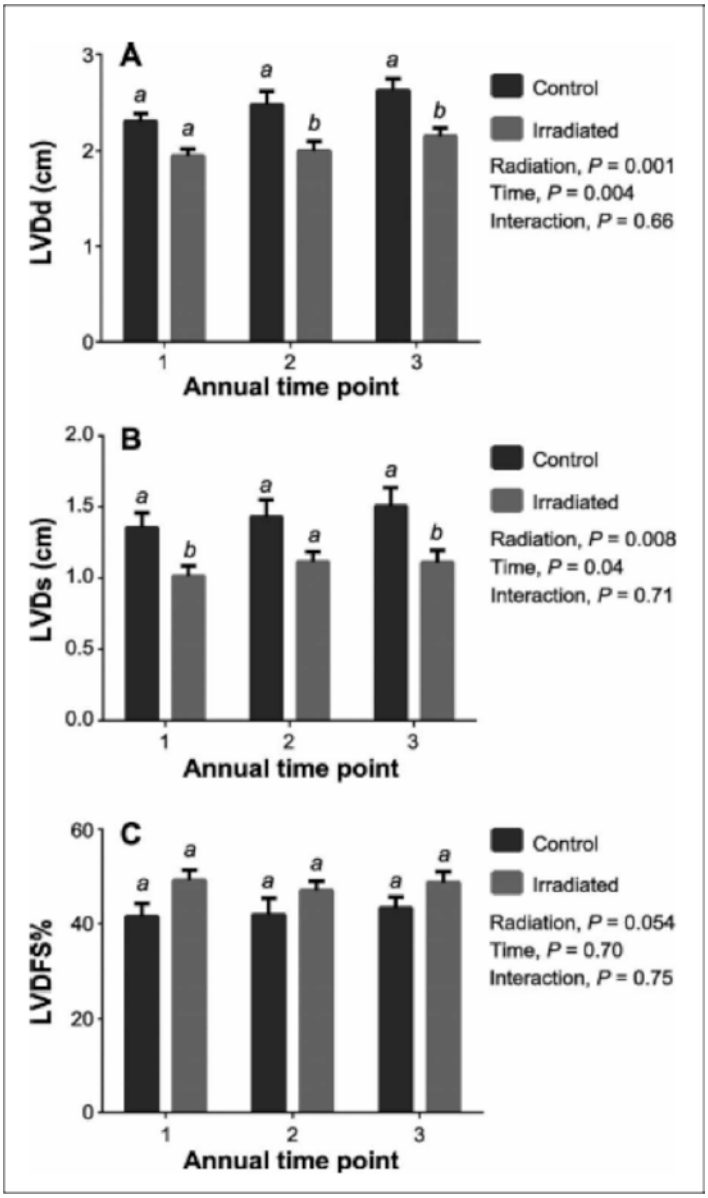


Figure 2.2. Effects of prior exposure on cardiac function via echocardiographic assessments. Panels A and B: Echo left ventricular diastolic and systolic diameters (LVDd and LVDs, respectively) were significantly smaller in irradiated animals (RMANOVA, P ¼ 0.001 and P¼0.008). Panel C: Fractional shortening percentage (LVDFS%) tended to be greater in irradiated animals (RMANOVA, P ¼ 0.054). Data are mean 6 SEM. Control (n=11); irradiated (n=20) across all years. Unlike letters denote significance between control and irradiated animals within time points with P values, 0.05.

Radiation had no effect on ejection fraction (EF%) (p=0.930, RMANOVA; data not shown). Irradiated animals tended to have higher mean annual heart rate (p=0.06, RMANOVA), but were not different from controls in systolic, diastolic, or mean arterial blood pressure (all p>0.35, Table 2.2).

Table 2.2: Effects of Prior Irradiation on Heart Rate and Blood Pressure in the Echocardiographic Subset of Animals.

	Time point 1		Time point 2		Time point 3		*P* value		
	Control	TBI	Control	TBI	Control	TBI	Time	TBI	Time × TBI
n =	11	21	11	21	11	21			
Heart rate (beats per min)	138.7	151	128	137	125	132	**0.0001**[a]	0.06	0.31
SEM	4.4	3.1	4.6	2.8	4.4	2.8			
Systolic BP (mm Hg)	128	127	126	123	126	127	0.067	0.83	0.27
SEM	2.9	2.8	2.9	2.6	2.9	3.1			
Diastolic BP (mm Hg)	68.2	73.3	67.4	69.9	70.8	72.4	0.10	0.36	0.42
SEM	1.8	2.3	3.3	2.0	2.4	2.5			
Mean BP (mm Hg)	91.6	94.4	88.6	90.8	91.6	92.3	0.06	0.57	0.73
SEM	2.3	2.4	3.2	2.1	1.9	2.5			

Notes. Time points 1, 2 and 3 correspond to years 1, 2 and 3. Data were evaluated by repeated measures ANOVA. SEM = standard error of the mean. BP = blood pressure.
[a] Significance ($P < 0.05$) indicated in bold face.

Biomarker assessments to date have shown clear evidence of a persistent systemic pro-inflammatory state (Figure 2.3). Monocyte chemoattractant protein 1 (MCP-1) and lipopolysaccharide binding protein (LBP) are persistently elevated in irradiated animals, relative to controls. Additional biomarkers are under development (Table 2.3) and have not yet shown significant differences. These include C-reactive protein (hsCRP), Interleukin 6 (IL-6), soluble cluster of differentiation 14 (sCD-14), and soluble intercellular adhesion molecule (sICAM).

Table 3.3 Effects of Prior Irradiation on Circulating Biomarkers in Control and Irradiated Animials.

	Control (n = 13)	TBI (n = 27)	*P* value
hsCRP (ng/ml)	986	1,507	0.12
SEM	227	198	
IL-6 (pg/ml)	1.95	4.25	0.12
SEM	0.46	0.96	
sCD-14 (ng/ml)	1,150	1,286	0.25
SEM	77	71	
sICAM (U/ml)	24.4	30.6	0.53
SEM	3.0	6.7	

Note. Data are mean ± standard error of mean (SEM).

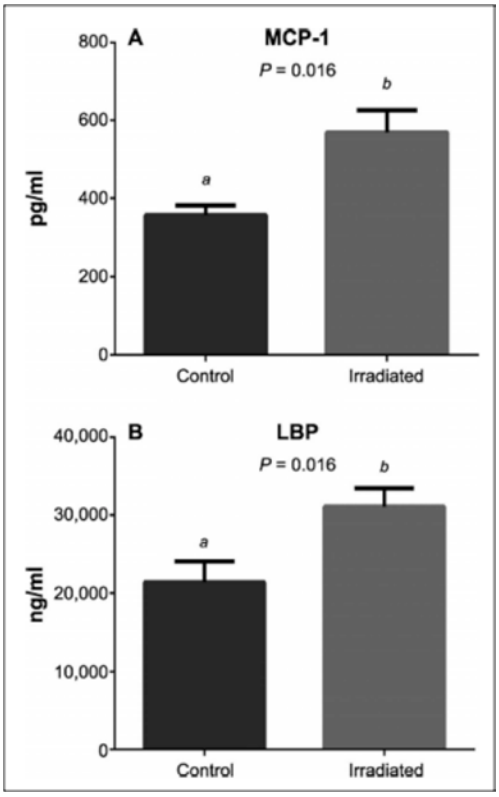


Figure 2.3: Effects of irradiation on circulating MCP-1 and LBP. Both markers are significantly higher in irradiated animals (t test, PO-0.016). Data are presented as mean ± SEM. Differing letters denote significant differences with p <0.05.

4) Other achievements: Abstract submitted to 2016 Radiation Research Meeting describing increased serum IL-10 and 15 in irradiated RSC monkeys and inverse relationships between these cytokines with left ventricular wall thickness and ejection fraction accepted for poster presentation.

Cardiac Biomarkers: Circulating troponin-I and nt-proBNP fragment (8-29) were below the detection limits of the ELISA assays, which were 0.156ng/mL for cardiac troponin-I and 171 pmol/L for nt-proBNP.

Relationships between biomarkers and other phenotypes: Circulating levels of MCP-1 were positively correlated with circulating levels of LBP (r=0.37, p=0.018, n=40). MCP-1 and LBP were not correlated with cardiac functional phenotypes (all p> 0.05, data not shown).

Diabetes, Body Weight, and Plasma Lipids: Across the sampled cohort of 59 subjects a high incidence of Type 2 diabetes mellitus (T2DM, defined as HgbA1c > 6.5%) was observed in the irradiated cohort (13 out of 46), compared to no subjects in the control animals (0 out of 13) (p=0.03, Chi-square). Diabetic subjects had significantly

higher HbA1c values than controls (p=0.0001) and irradiated non-diabetic monkeys (p=0.0001), while controls were not different from irradiated non-diabetic monkeys (p=0.9) (Table 2.4). Body weight and plasma lipids were influenced by radiation and diabetes status. Irradiated non-diabetic animals had significantly lower body weights than Control animals and irradiated diabetic animals (all p<0.05). TBI subjects with diabetes exhibited higher TG than controls (p<0.005) or TBI non-diabetic monkeys (p=0.003), and had lower HDLc compared to control (p<0.03) and non-diabetic irradiated monkeys (p<0.002). Further details regarding the pathophysiology of T2DM in irradiated macaques are published elsewhere (Kavanagh et al., 2015).

Table 4: Effects of Prior Radiation Exposure and Diabetes on Plasma Lipids in the Echocardiographic Subset of Animals.

	Control (n = 11)	TBI, no T2DM (n = 13)	TBI + T2DM (n = 8)	ANOVA *P* value	*P* values: Control vs. TBI, no T2DM	Control vs. TBI + T2DM	TBI, no T2DM vs. TBI + T2DM
HbA1c (%)	4.5	4.7	9.6	**0.0001**[a]	0.9	**0.0001**[a]	**0.0001**[a]
SEM	0.1	0.1	0.6				
Body weight (kg)	12.9	8.8.	14.2	**0.0001**[a]	**0.0004**[a]	0.45	**0.0001**[a]
SEM	1.0	0.4	0.7				
TPC (mg/dl)	164	187	213	0.19	0.57	0.16	0.57
SEM	18	9	30				
TG (mg/dl)	88.9	76.5	484	**0.002**[a]	0.99	**0.005**[a]	**0.003**[a]
SEM	9.1	3.5	179				
HDL (mg/dl)	71.6	80.6	47.8	**0.003**[a]	0.49	**0.03**[a]	**0.002**[a]
SEM	7.6	4.1	5.9				

Notes. All lipid measurements were performed at a single time point. All lipids were evaluated using an unpaired two-tailed *t* test and ANOVA to evaluate the effect of T2DM on lipid measurements. Between group ANOVA *P* values. Post hoc analyses corrected for multiple comparisons via Tukey's HSD. TBI = total-body irradiation; T2DM = type 2 diabetes mellitus; SEM = standard error of the mean.

[a] Significance ($P < 0.05$) indicated in bold face.

Effects of Diabetes on Individual Phenotypes: Cardiac phenotypes (ventricular diameters, heart rate, and blood pressure) and non-lipid biomarkers did not differ between diabetic and non- diabetic irradiated animals (data not shown), and HgbA1c concentrations were not correlated with cardiac phenotypes (all p>0.05), but were associated with body weight in animals in the echocardiographic subset (all p<0.01).

Conclusions: These results demonstrate that single whole body doses of 6.5-8.4 Gy of radiation produced long-lasting effects including a high incidence of myocardial fibrosis, reduced left ventricular diameter, and elevated systemic inflammation. The studies are limited because we had not pre-irradiation phenotypes or blood samples to provide a background basis for individual animal variability. This limitation will be overcome in the prospective cohort.

Prospective Cohort Baseline Data Collections:

Echocardiographic data: We collected baseline echocardiographic data from 20 animals in the prospective prior to radiation exposure. Figure 2.4 illustrates some of the baseline structural parameters obtained from the prospective cohort. Additional echocardiographic measurements will be obtrained immediately post-irradiation revcovery.

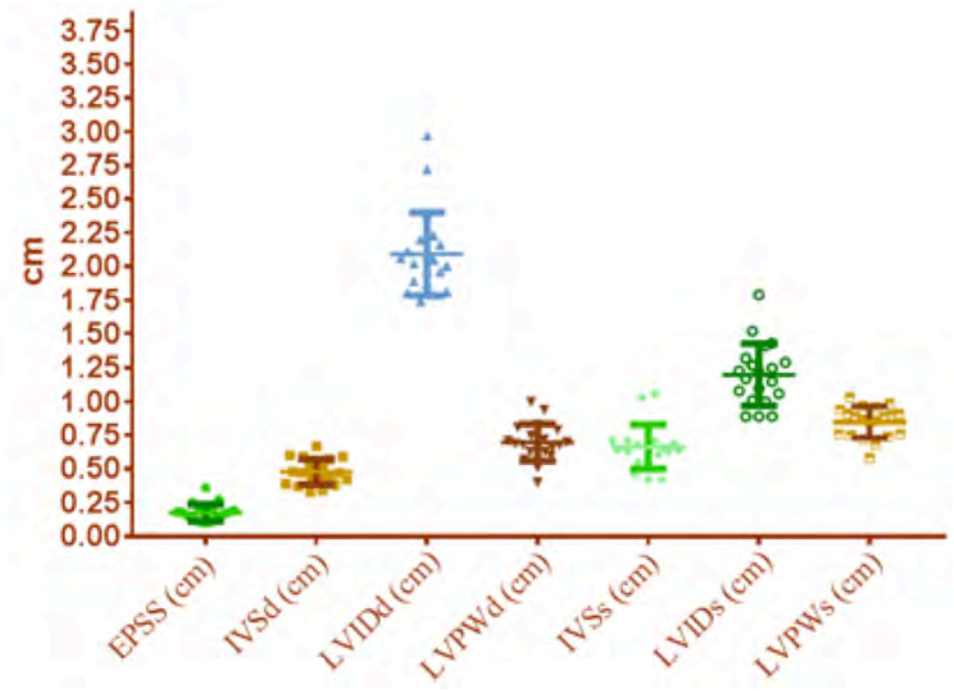


Figure 2.4: Baseline Cardiac Structural Parameters from Echocardiographic Assessments of the Prospective Cohort. EPSS: E-point septal separation, distance from the anterior mitral valve leaflet and ventricular septum in early diastole (d), IVS: interventricular septum, LVID: left ventricle internal diameter, LVPW: left ventricle posterior wall thickness.

Preliminary Cytoline Profiling in the Radiation Survivor Cohort: Serum samples were assessed using multiplex biomarker profiling. Previously irradiated monkeys (n=70) had significantly elevated serum levels of interleukin-10 (IL-10) and interleukin-15 (IL-15) (both $p<0.03$) relative to a companion cohort of non-irradiated male rhesus monkeys (n=14). Within irradiated animals, serum levels of IL-10 and IL-15 were inversely associated with left ventricular diameters, which had previously been shown to be significantly smaller in irradiated animals (DeBo et al., Rad Res 2016). In addition, IL-15 was inversely associated with left ventricular posterior wall thickness ($p<0.05$) and ejection fraction ($p<0.05$). Among irradiated animals, serum IL-10 and IL-15 levels were similar among diabetic and non-diabetic monkeys and were not correlated with hemoglobin A1c levels. The results suggest long lasting effects of TBI on circulating IL-10 and IL-15, both of which were associated with radiation sensitive cardiac phenotypes. This data was presented as a poster at the 2016 Radiation Research Meeting.

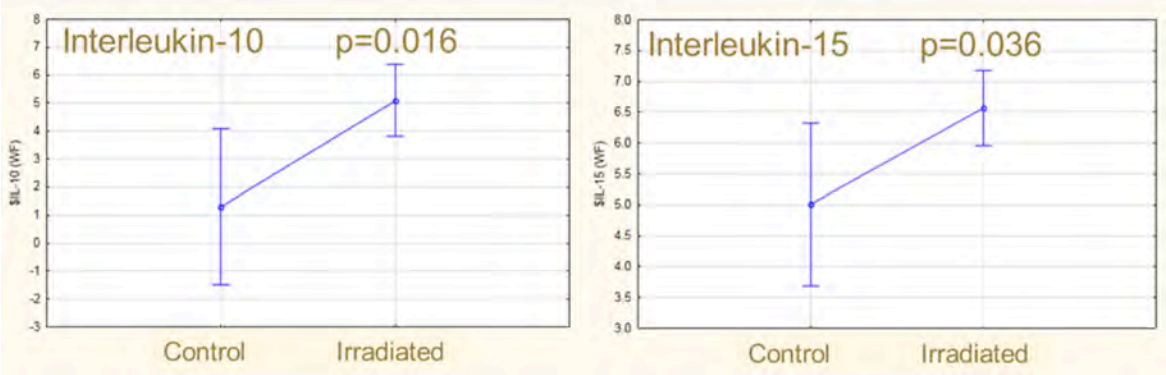


Figure 2.5: Serum IL-10 and IL-15 in Control and Irradiated Macaques from the RSC. Preliminary data indicate that these additional cytokine measures reflect systemic inflammation, as for MCP-1 and LBP previously.

Project 3: Immune Recovery (Sempowski/Chen)

1) Major Activities

a. We have confirmed with the Duke IACUC that an IACUC animal protocol is not required by Duke for the NHP studies being performed at Wake Forest.
b. Duke IACUC animal protocol was approved by ACURO (Task 2-4).
c. Amendments to the approved animal protocol was approved by both Duke IACUC and ACURO.
d. Optimization and planning of NHP Studies; generation of baseline flow cytometry data and comprehensive immunophenotype panels. Collaborative effort with Project 2 to add macrophage subsets to analysis (long-term/prospective cohorts). All logistics regarding sample transfer from WF to Duke have been finalized. The Sempowski lab (Project 3) will process and freeze PBMC for Project 4 (Dave) to minimize cost and blood draw impact on animals.
e. Ongoing murine studies to study immune recovery post-irradiation.

2) Specific Objectives

As noted above (Page 8)

3) Results/Key Outcomes

SA1 Major Task 1: NHP Studies (Sempowski, Duke)

Worked closely with Wake Forest NHP core team and Project 4 (Dave) to determine sample collection needs (tube types, volumes) and timeline for both the Long-term Survivor NHP cohort (X-10-12) and the Prospective NHP Cohort (X-15-03) to accomplish the studies planned for year 1 under Task 1. These preparative meetings and teleconferences regarding radiation dosimetry, sample collection volume and timing, vaccination and possible Flu challenge were essential to coordinate all center studies that relied on these two NHP cohorts. Sample collection

and analysis for Project 3, Task 1, were initiated in Year 1 and will continue through 60 months per SOW.

Critical to the success of project 3 and 4 was to work through the logistics of fresh NHP blood collection at the WF primate center and transportation of the whole blood to Duke (~2 hr drive) for central processing (Sempowski Lab). A series of pilot studies were performed to assess viability of cells using first overnight Federal Express Shipment (room temp) versus staff driving the samples from WF to Duke. It was determined that viability was equivalent with Overnight Federal Express shipment and that this was most efficient for all parties.

Based on pre-determined sample collection and shipment schedules (coordinated by WF NHP Core team) fresh NHP blood (Two, 10ml GTT sodium heparin tubes) is packaged and shipped for first overnight Federal Express shipment (Room Temp) to the Sempowski lab at Duke. Blood receipt is confirmed to WF, processed by standard ficoll separation technique to isolate peripheral blood mononuclear cells (PBMC) and plasma. Aliquots of PBMC are retained for a fresh immunophenotype and viability assessment (see below) and the remainder of the PBMC are cryopreserved using standard DMSO freeze medium and stored in Liquid Nitrogen for batch processing for sjTREC and possibly TCR sequencing. Aliquots of Plasma are also crypopreserved for potential add on studies. All samples and aliquots are inventoried in the Sempowski group FreezerPro lab inventory control system. All freezers are on 24/7 emergency power back up and 24/7 remote temperature monitoring (Minus 80 Inc.) with alarm notifications via phone, text, email. An empty back up -80°C freezer is kept at the ready in case a freezer goes down. The Sempowski lab is housed in the Duke Regional Biocontaiment laboratory, which has an external Bulk Liquid Nitrogen (LN_2) storage tank that contains sufficient LN_2 to supply all storage tanks for 1 month without refill.

Optimization of Initial Rhesus PBMC Immunophenotype Panel

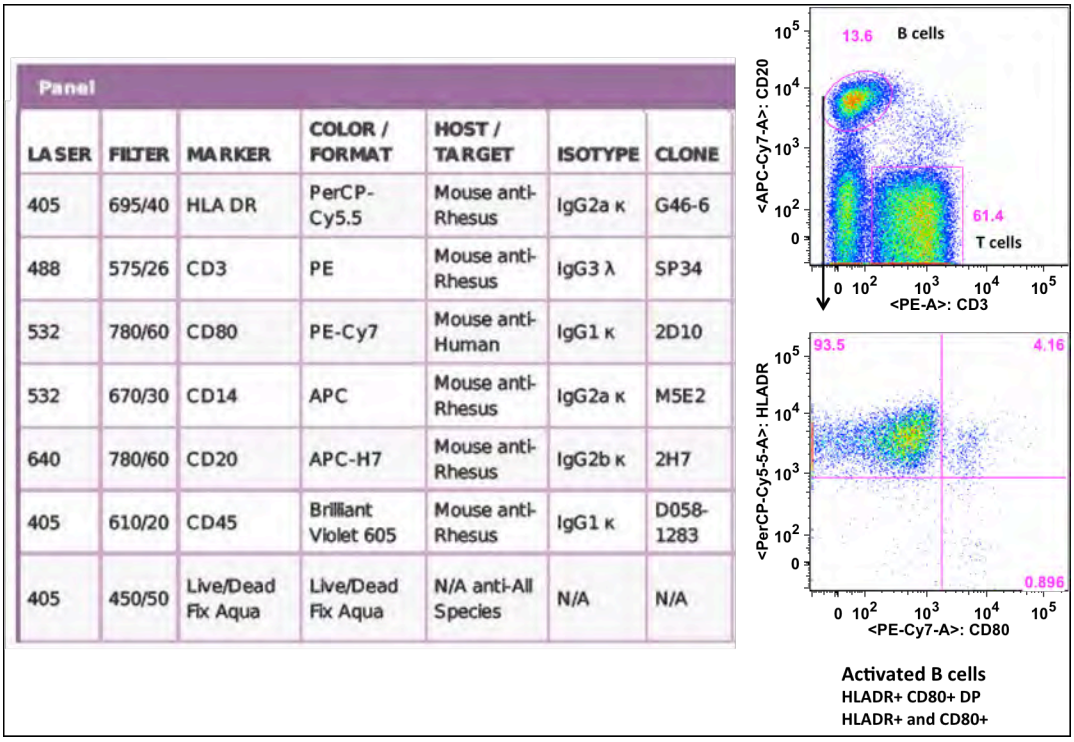
Three pilot studies were performed in year 1 to develop our initial immuophenotype panel to define Rhesus NHP PBMC cell subsets. To minimize blood draws on the WF NHP cohorts, we elected to purchase NHP blood from a commercial vendor (AllCells). PBMCs were isolated per lab SOPs and surface stained with specific panels of directly conjugated antibodies (see **Table 3.1**). Stained cells were fixed with 0.4% PFA and then analyzed on one of our 18-color BD LSRII flow cytometers. Titrations were performed for all reagents to optimize antibody volumes and dilutions to minimize spectral overlap and maximize surface marker detection. Non-reactive and dimly reactive clones were eliminated and replaced. All immunophenptype sample data (FCS 3.0 files) are stored in a secure Collaborative Data Management System run by the Duke Human Vaccine Institute Flow Cytometry Facility (Biotrue). Files are downloaded and analyzed in FlowJo software (Treestar Inc.). Data files and reports are shared with WF collaborators via the Secure DukeBox file sharing portal.

Table 3.1: Initial Immunophenotype Panel for Project 3

		FLUOR							
Panel#	Subsets Identified	FITC	PerCP-Cy5.5	PE	PE-Cy7	APC	APC-Cy7	BV421	BV605
1	T, B, Monos, Activated B, Monos		HLADR	CD3	CD80	CD14	CD20	Live/Dead	CD45
2	CD4 & CD8 T Naïve & Memory, B cells	CD45RA	CD4		CD8			Live/Dead	CD45
3	DCs	CD1c		CD11c		CD3/CD20 dump	HLADR	Live/Dead	CD45
4	T, NK, NK-T			CD16		CD3		Live/Dead	CD45
5	Megakaryocyte and Platelet marker	CD31	CD41					Live/Dead	CD45
6	Monocyte subsets (M1/M2)			CD14		CD16		Live/Dead	CD45

Below are a series of figures (**Figures 3.1-3.6**) showing the instrument configuration to detect the specific fluorescent labels, the markers detected and the mAb reagents used in each of the immnophenotype panels (**Table 3.1**). In addition the figures show representative dot plots, gate strategies and the target subsets. A fixable aqua vital dye is used in all sampels to identify live vs dead cells. CD45 is also included as a pan-hematopoietic marker.

Figure 3.1: Panel 1 - Single Cells / Live-Dead / CD45+ Lymph gate / CD3 vs CD20 / CD80 vs HLADR

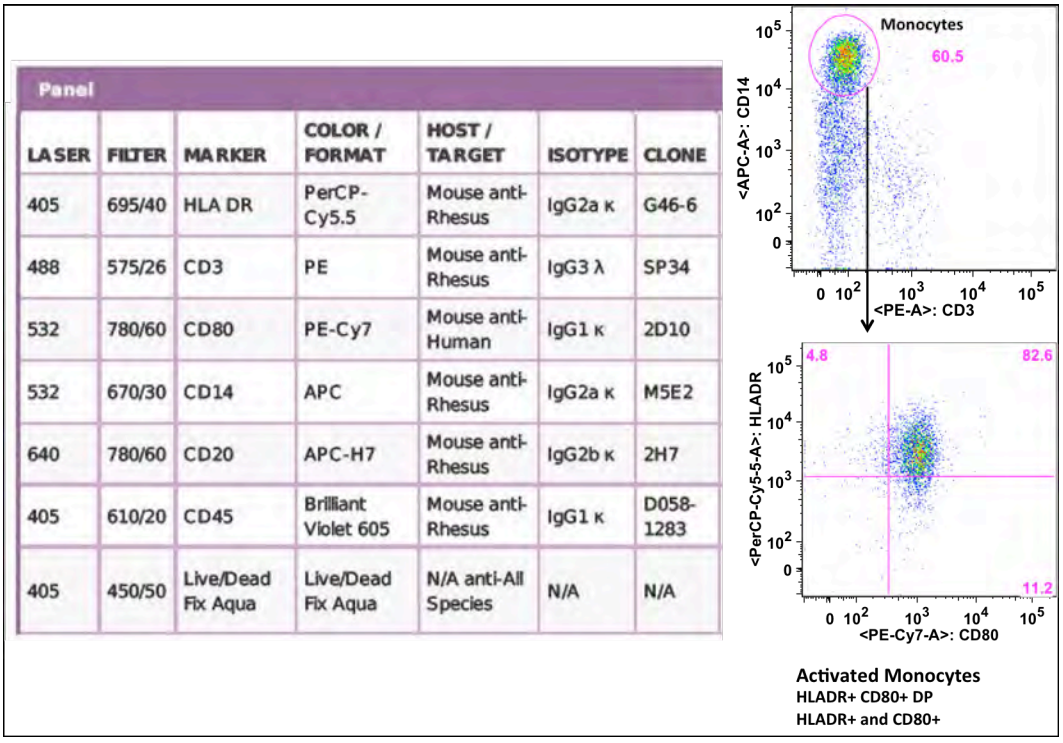


Panel

LASER	FILTER	MARKER	COLOR / FORMAT	HOST / TARGET	ISOTYPE	CLONE
405	695/40	HLA DR	PerCP-Cy5.5	Mouse anti-Rhesus	IgG2a κ	G46-6
488	575/26	CD3	PE	Mouse anti-Rhesus	IgG3 λ	SP34
532	780/60	CD80	PE-Cy7	Mouse anti-Human	IgG1 κ	2D10
532	670/30	CD14	APC	Mouse anti-Rhesus	IgG2a κ	M5E2
640	780/60	CD20	APC-H7	Mouse anti-Rhesus	IgG2b κ	2H7
405	610/20	CD45	Brilliant Violet 605	Mouse anti-Rhesus	IgG1 κ	D058-1283
405	450/50	Live/Dead Fix Aqua	Live/Dead Fix Aqua	N/A anti-All Species	N/A	N/A

Figure 3.2: Panel 1 - Single Cells / Live-Dead / CD45+ Monocyte gate / CD3 vs CD14 / CD80

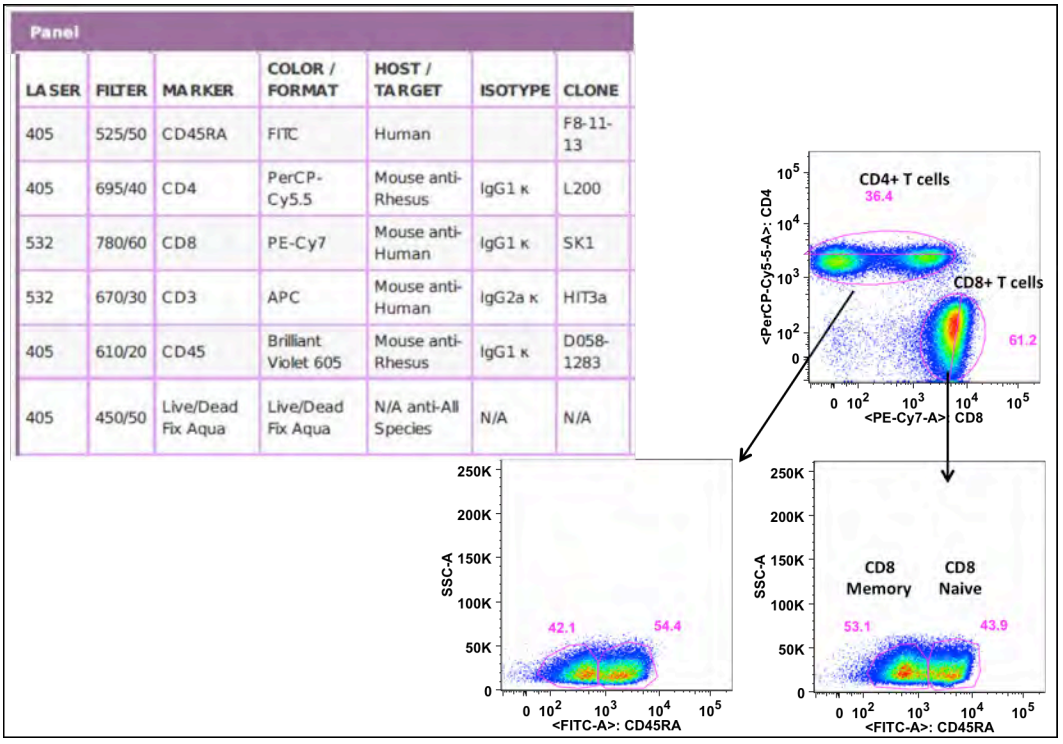
vs HLADR



Panel

LASER	FILTER	MARKER	COLOR / FORMAT	HOST / TARGET	ISOTYPE	CLONE
405	695/40	HLA DR	PerCP-Cy5.5	Mouse anti-Rhesus	IgG2a κ	G46-6
488	575/26	CD3	PE	Mouse anti-Rhesus	IgG3 λ	SP34
532	780/60	CD80	PE-Cy7	Mouse anti-Human	IgG1 κ	2D10
532	670/30	CD14	APC	Mouse anti-Rhesus	IgG2a κ	M5E2
640	780/60	CD20	APC-H7	Mouse anti-Rhesus	IgG2b κ	2H7
405	610/20	CD45	Brilliant Violet 605	Mouse anti-Rhesus	IgG1 κ	D058-1283
405	450/50	Live/Dead Fix Aqua	Live/Dead Fix Aqua	N/A anti-All Species	N/A	N/A

Figure 3.3: Panel 2 - Single Cells / Live-Dead / CD45+ Lymph gate / CD3+ /CD4 vs CD8 / CD45RA from CD4 & CD8



Panel

LASER	FILTER	MARKER	COLOR / FORMAT	HOST / TARGET	ISOTYPE	CLONE
405	525/50	CD45RA	FITC	Human		F8-11-13
405	695/40	CD4	PerCP-Cy5.5	Mouse anti-Rhesus	IgG1 κ	L200
532	780/60	CD8	PE-Cy7	Mouse anti-Human	IgG1 κ	SK1
532	670/30	CD3	APC	Mouse anti-Human	IgG2a κ	HIT3a
405	610/20	CD45	Brilliant Violet 605	Mouse anti-Rhesus	IgG1 κ	D058-1283
405	450/50	Live/Dead Fix Aqua	Live/Dead Fix Aqua	N/A anti-All Species	N/A	N/A

Figure 3.4: Panel 3 - Single Cells / Live-Dead / CD45+ Mono-Gran gate / CD3 CD20 dump / HLADR+ / CD1c vs CD11c

Dump Channel

LASER	FILTER	MARKER	COLOR / FORMAT	HOST / TARGET	ISOTYPE	CLONE
532	670/30	CD3	APC	Mouse anti-Human	IgG2a κ	HIT3a
532	670/30	CD20	APC	Mouse anti-Human	IgG2b κ	2H7

Panel

LASER	FILTER	MARKER	COLOR / FORMAT	HOST / TARGET	ISOTYPE	CLONE
405	525/50	CD1c	FITC	Mouse anti-Human	IgG1 κ	L161
488	575/26	CD11c	PE	Mouse anti-Human	IgG1 κ	3.9
640	780/60	HLA DR	APC-H7	Mouse anti-Human	IgG2a κ	G46-6
405	610/20	CD45	Brilliant Violet 605	Mouse anti-Rhesus	IgG1 κ	D058-1283
405	450/50	Live/Dead Fix Aqua	Live/Dead Fix Aqua	N/A anti-All Species	N/A	N/A

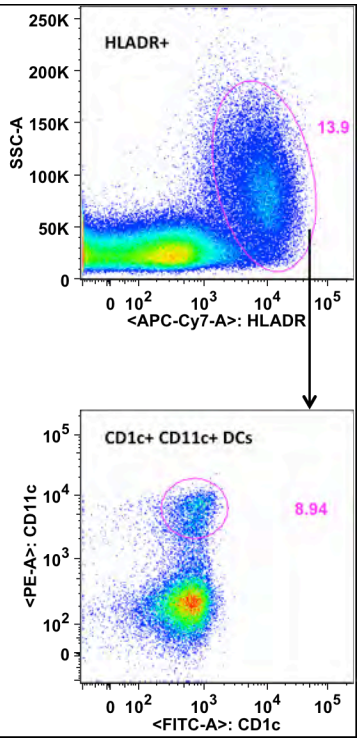


Figure 3.5: Panel 4 - Single Cells / Live-Dead / CD45+ Lymph gate / CD3 vs CD16

Panel

LASER	FILTER	MARKER	COLOR / FORMAT	HOST / TARGET	ISOTYPE	CLONE
488	575/26	CD16	PE	Mouse anti-Rhesus	IgG1 κ	3G8
532	670/30	CD3	APC	Mouse anti-Human	IgG2a κ	HIT3a
405	610/20	CD45	Brilliant Violet 605	Mouse anti-Rhesus	IgG1 κ	D058-1283
405	450/50	Live/Dead Fix Aqua	Live/Dead Fix Aqua	N/A anti-All Species	N/A	N/A

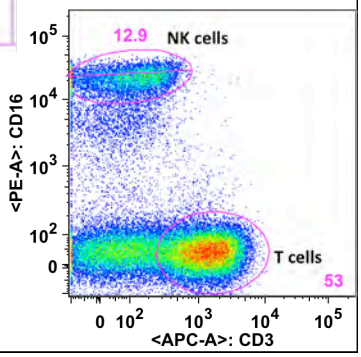
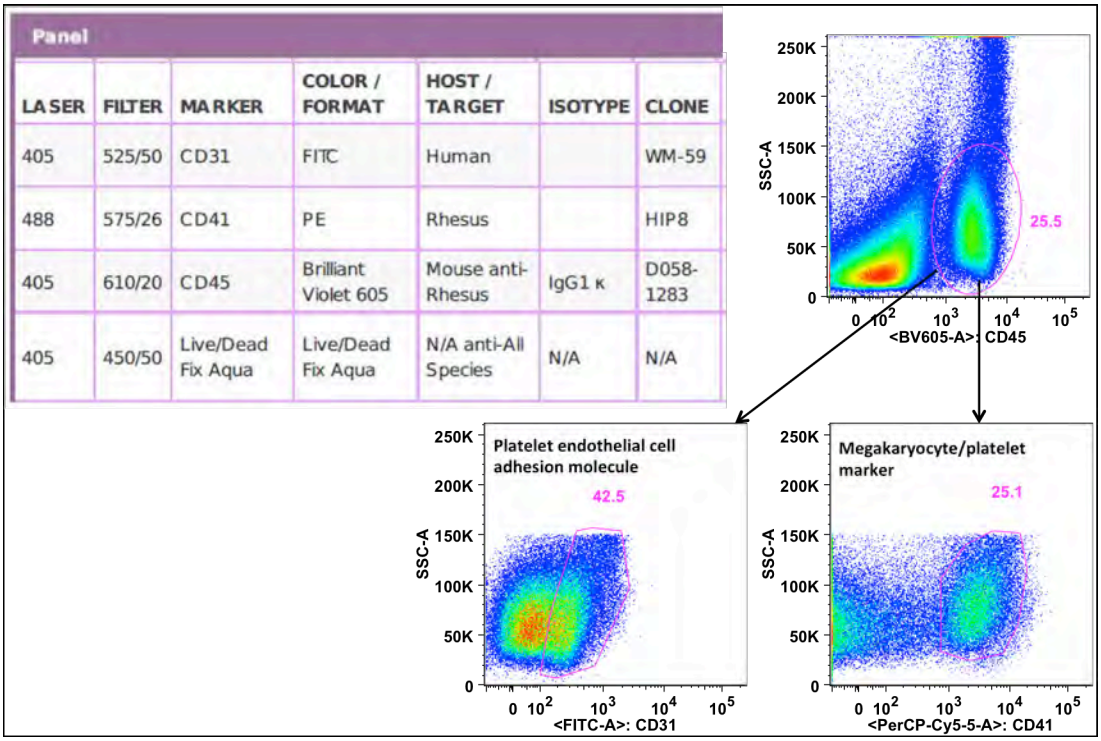


Figure 3.6: Panel 5 - Single Cells / Live-Dead / CD45+ Lymph gate / CD31 and CD41

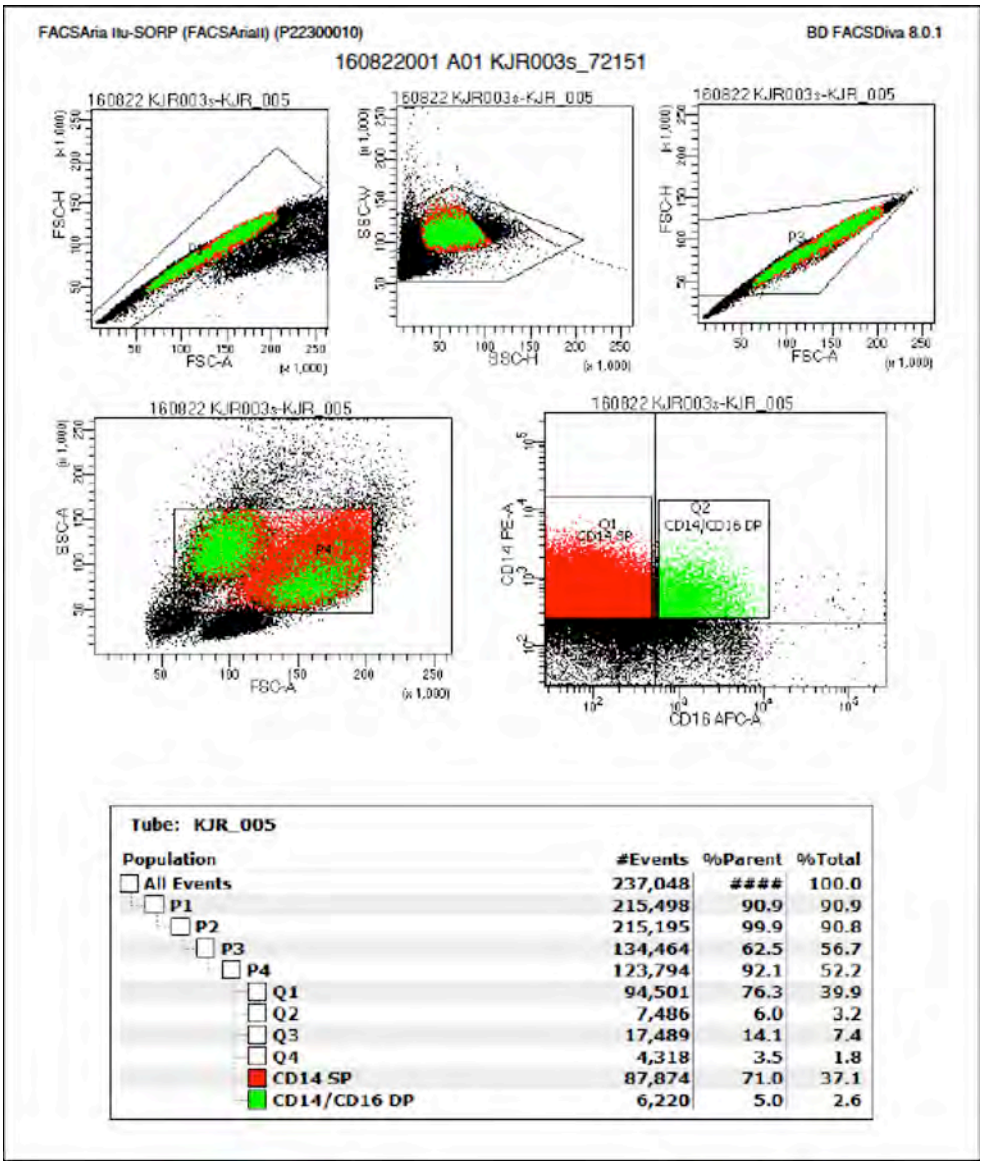


Panel

LASER	FILTER	MARKER	COLOR / FORMAT	HOST / TARGET	ISOTYPE	CLONE
405	525/50	CD31	FITC	Human		WM-59
488	575/26	CD41	PE	Rhesus		HIP8
405	610/20	CD45	Brilliant Violet 605	Mouse anti-Rhesus	IgG1 κ	D058-1283
405	450/50	Live/Dead Fix Aqua	Live/Dead Fix Aqua	N/A anti-All Species	N/A	N/A

Panel 6 was added at the request of Dr. Register for project #2 to quantify and isolate (sort) monocyte populations (CD14 single positive and CD14/16 double positive. From Aug 22-Sept 1, 2016 Dr. Register prepared CD14-enriched preparations of monocytes stained with Panel 6 from 16 NHP from the WF cohort. The samples were brought to Duke for fluorescent-activated cell sorting on our 18 color BD FACS Aria11 cell sorter. This machine is contained within a bioprotect hood and housed in a BSL2/3 containment facility (Duke Regional Biocontainment Laboratory). This allows for subset sorting of unfixed NHP samples that could potentially contain infectious agents. A representative FACS plot, gate strategy and sort results are shown in **Figure 3.7**. Sorted cells were spun down at Duke and processed for nucleic acid isolation and provided to Dr. Register's postdoc. CD45 and a live dead marker was include in the final immunophenotype panel to be deployed for Project 3 studies.

Figure 3.7: Panel 6 and M1 and M2 NHP sort scheme.



Baseline analysis of Prospective Cohort (X-15-03):

The X-15-03 prospective cohort of 20 NHP were irradiated in Year 1. Prior to irradiation we requested timepoint 1 (Baseline) peripheral blood samples. These samples, as well as future samples from defined time points over the 5-year study, were or will be processed as follows in Task 1 to monitor immune recovery:

Phenotypic analyses

- Flow cytometry phenotyping of peripheral blood populations
  - CBC with differential
  - PBMC isolated and phenotyped (n=20)

    Stained for T cells, B cells, dendritic cells, monocytes, platelets, NK, NK-T as described above and shown below

Molecular Analysis

- T cell receptor rearrangement analysis (sjTREC)
  - PBMC cryopreserved from all 20 NHP for batch analysis in year 2-5
- T cell receptor deep sequencing (when available or with Dave Project)
  - PBMC cryopreserved from all 20 NHP for batch analysis in year 2-5

Time point 1, or baseline, blood samples were taken for Project 3 from all prospective cohort animals in year 1. The age distribution is shown in **Figure 3.8**. Absolute numbers of red cells, white cells and platelets per ul whole blood are shown in **Figure 3.9**. Baseline % of white cell subsets (differential) are shown in **Figure 3.10.** Shown in **Figure 3.11** are the baseline measures of hemoglobin, HCT, mean corpuscular Hb, mean corpuscular volume and mean corpuscular Hb concentration.

Using our immunophenotype panel we have defined the % or frequency of our target peripheral blood cell subsets at Baseline (Time point 1) in the prospective cohort. **Figure 3.12** shows the baseline frequency in all 20 animals of myeloid DCs, T, B, NK and NK-T cells, and activated B cell subsets. **Figure 3.13** shows the baseline frequency in all 20 animals of CD4 and CD8 T cells and their respective naïve and memory populations.

Figure 3.8: Age at baseline in the prospective cohort (Time Point 1).

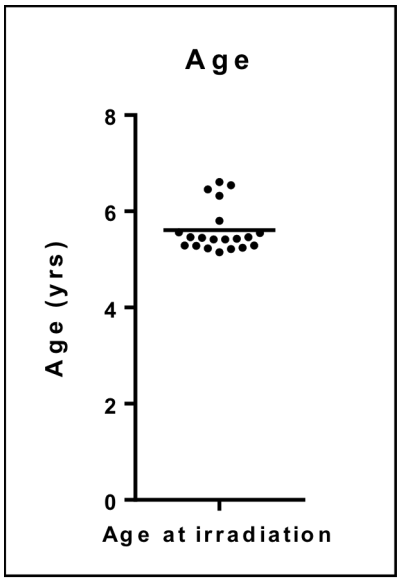


Figure 3.9: CBC at baseline in the prospective cohort (Time Point 1).

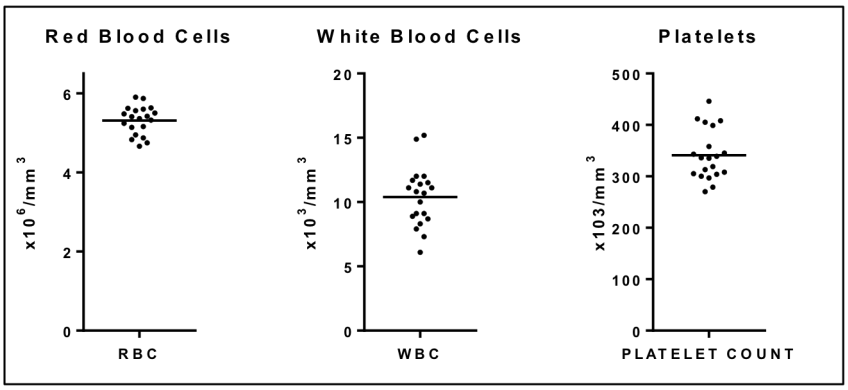


Figure 3.10: White cell differential at baseline in the prospective cohort (Time Point 1).

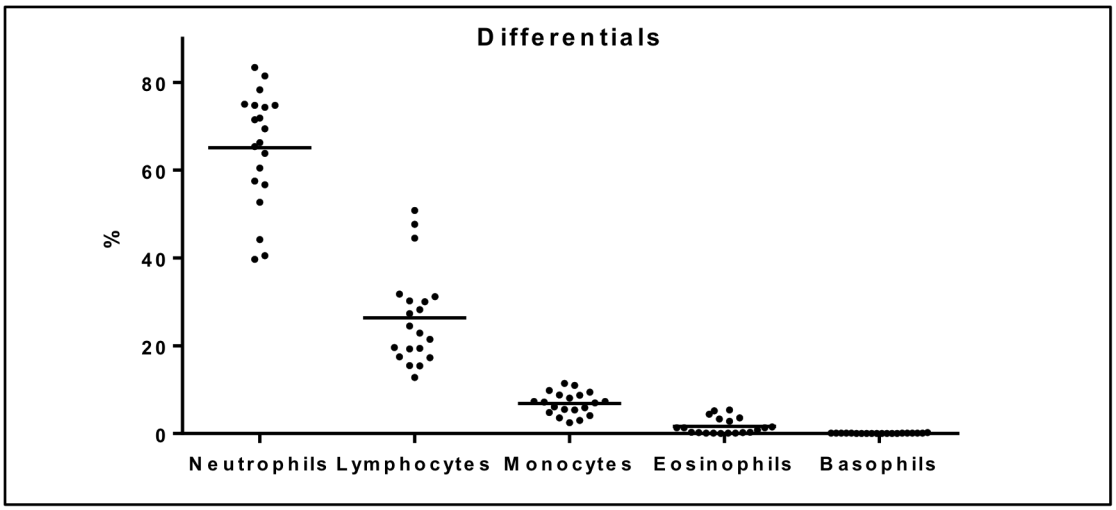


Figure 3.11: Additional CBC measures at baseline in the prospective cohort (Time Point 1).

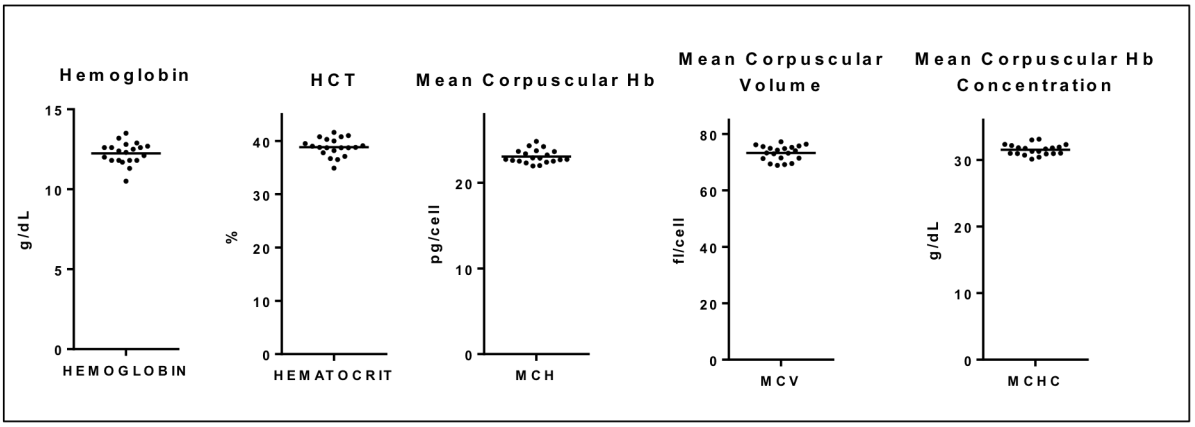


Figure 3.12: Frequency at baseline in the prospective cohort of myeloid DCs, T, B, NK and NK-T cells, and activated B cell subsets.

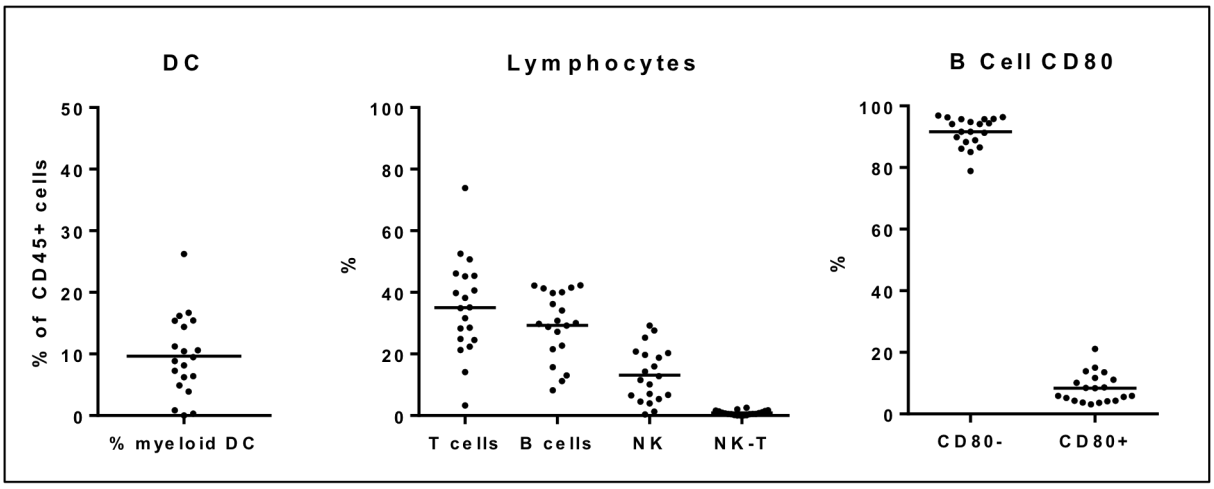
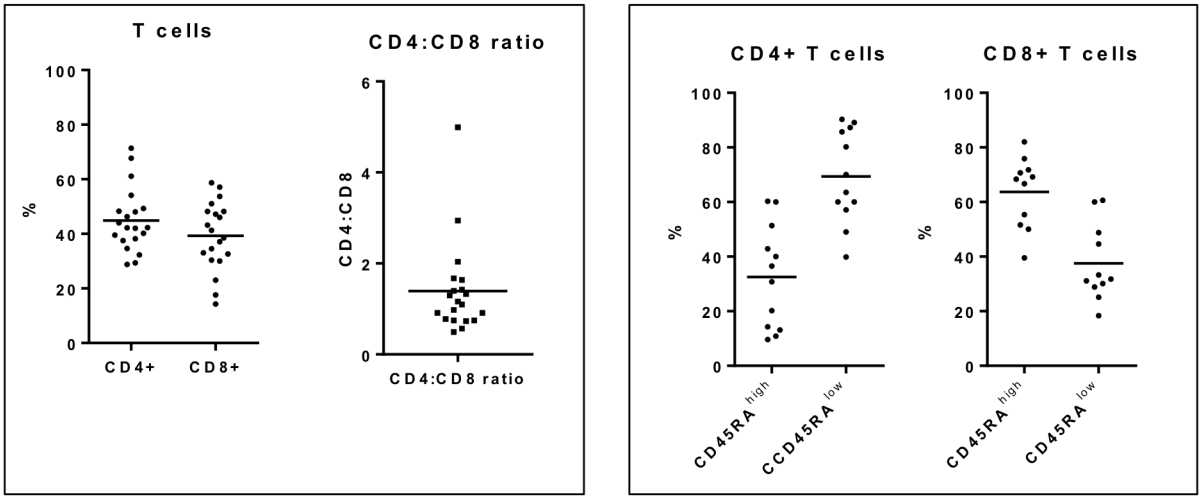


Figure 3.13: Frequency at baseline in the prospective cohort of of CD4 and CD8 T cells and their respective naïve and memory populations.



Enhanced Rhesus PBMC Immunophenotype Panel – Version 2.0:

Based on the analysis of the 20 prospective animals by flow cytometry and a review of the immune recovery literature, we have decided to develop an enhanced immunophenotype pane (Version 2.0). The improved NHP phenotyping staining panels are currently being optimized and will allow comparison to original panel described above and also allow us to further define the frequency of the following:

- Memory B cells
- Plasmacytoid and myeloid DC
- Central and effector memory T cells
- Cytolytic and non-cytolytic NK
- Classical/non-classical moncytes

Table 3.2: Enhanced Immunophenotype Panel for Project 3 – VERSION 2.0

	FITC	PerCP-Cy5.5	PE	PE-Cy5	PE-Cy7	APC	APC	APC-Cy7	BV605	
B cells	CD27	HLA-DR	CD3		CD80	CD14	live/dead	CD20	CD45	
T cells	CD28	CD4	CD45RA	CD95	CD8	CD3	live/dead		CD45	
DC	CD1c	CD14	CD11c		CD123	CD20 & CD3	live/dead	HLADR	CD45	
NK / NK-T	CD56	HLA-DR	CD16		CD8	CD3	live/dead	CD20	CD45	
Platelets / monocytes	CD31	CD41	CD16			CD14	live/dead	HLA-DR	CD45	

Molecular assessments of thymopoiesis (Both NHP Cohorts):

sjTREC analysis is a real-time PCR assay run on a 96-well assay platform. It is best to isolate CD4 and CD8 T cells from cryopreserved PBMC in batches with multiple time points. We plan to run the first batches of sjTREC in Year 2.

Cross-sectional analysis of Long-term Cohort (X-10-12):

The X-10-12 Long-term Survivor cohort of 108 NHP (control and irradiated) will be cross-sectionally analysed at distinct dates over the 5 year study to monitor immune recovery and correlate with radiation dose and age. As with the prospective cohort we plan to perform the below analyses. The first series of 108 samples are scheduled to be shipped and processed in November 2016 and the results will be included in the first quarterly report of Year 2.

Phenotypic analyses

- Flow cytometry phenotyping of peripheral blood populations
  - CBC with differential
  - PBMC isolated and phenotyped (n=108)
    Stained for T cells, B cells, dendritic cells, monocytes, platelets, NK, NK-T as described above

Molecular Analysis

- T cell receptor rearrangement analysis (sjTREC)
  - PBMC cryopreserved from all 20 NHP for batch analysis in year 2-5
- T cell receptor deep sequencing (when available or with Dave Project)
  - PBMC cryopreserved from all 20 NHP for batch analysis in year 2-5

Major Task 2: Murine Studies (Chen, Duke)

The initial experiment to determine the roles of thymopoiesis and peripheral expansion in overall T cell recovery in the classic radiation injury mouse model have been completed. The data are presented below:

T cell recovery post-irradiation is radiation dose-dependent in mouse peripheral blood:

As a first step, we studied the effect of radiation dose on overall T cell recovery after mice received different doses of radiation. C57BL/6 mice were irradiated with three different doses of radiation (2, 5, 7 Gy), Total T cells (CD3+) and B cells (B220+) were measured in peripheral blood over time post-irradiation. As shown in **Figure 3.14,** both T and B cells dropped rapidly in irradiated mice at a radiation dose-dependent manner at day +3 after irradiation. Recovery of both T and B cells started after day +14 post-irradiation. Both T and B cells in the 2 Gy group recovered back to normal within 28 days after irradiation. T and B cells did not recover back to normal until 56 and 180 days respectively in the 5 Gy group. Neither T nor B cells recovered back to normal during the observation

period of 180 days in the 7 Gy group.

As shown in **Figure 3.15 and 3.16**, the recovery of total CD4 and CD8 T cells followed a very similar pattern as total T cells (**Figure 3.14**) except CD8 T cell at the 5 Gy group did not recover back to normal at day +180. Similar results were obtained for both CD4+ and CD8+ naive (TN) and effector memory (TEM) T cells. Both CD4+ and CD8+ central memory (TCM) T cells were able to recover back to normal in all three irradiation groups. The numbers of TCM were even higher than normal in the 2 Gy and 5 Gy groups.

Figure 3.14: Immune cell recovery post-irradiation is radiation dose-dependent in peripheral blood.

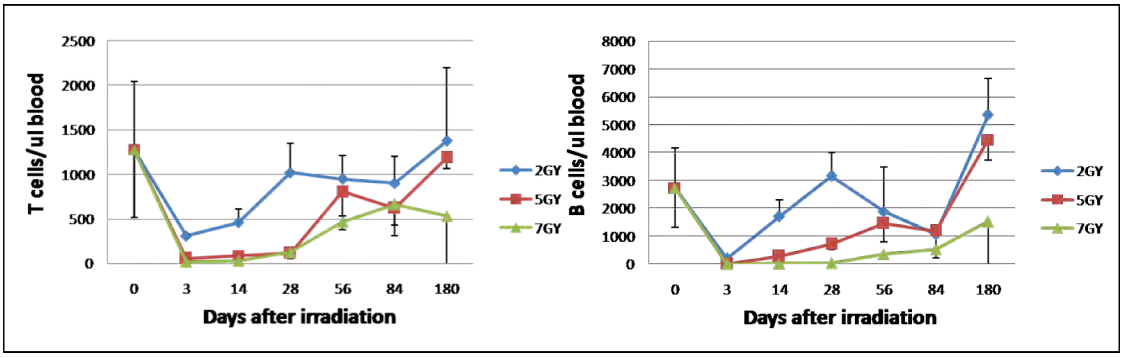


Figure 3.15: CD4 T cell recovery post-irradiation is radiation dose-dependent in peripheral blood.

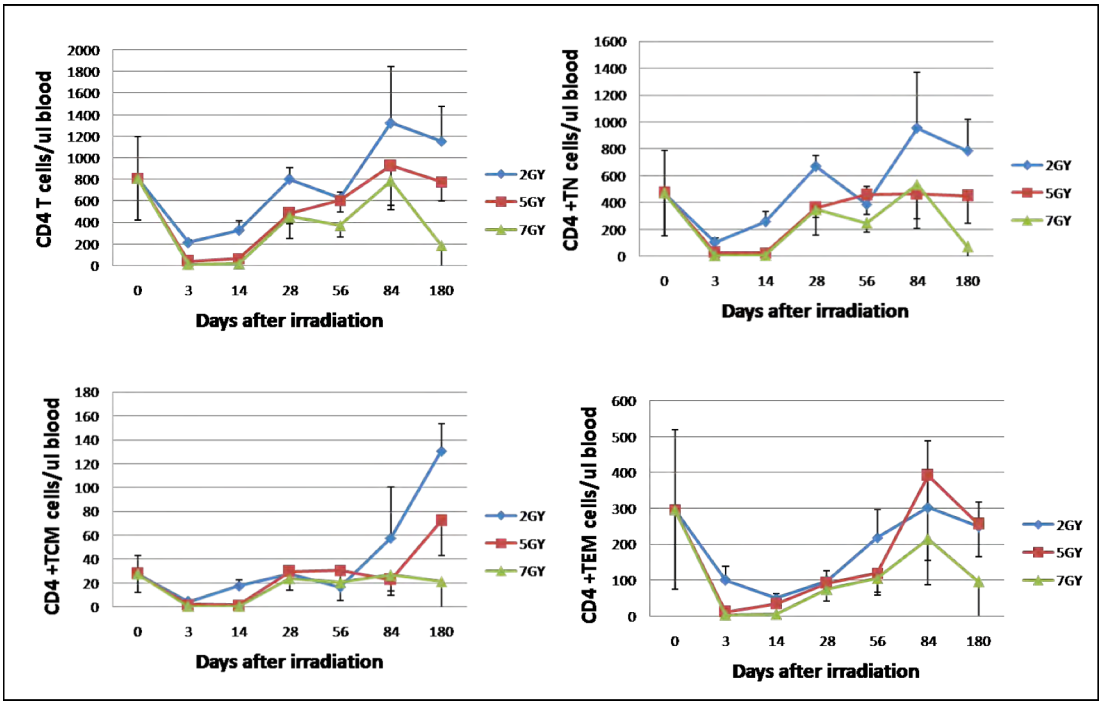


Figure 3.18: CD4 T cell recovery post-irradiation is radiation dose-dependent in mouse spleen

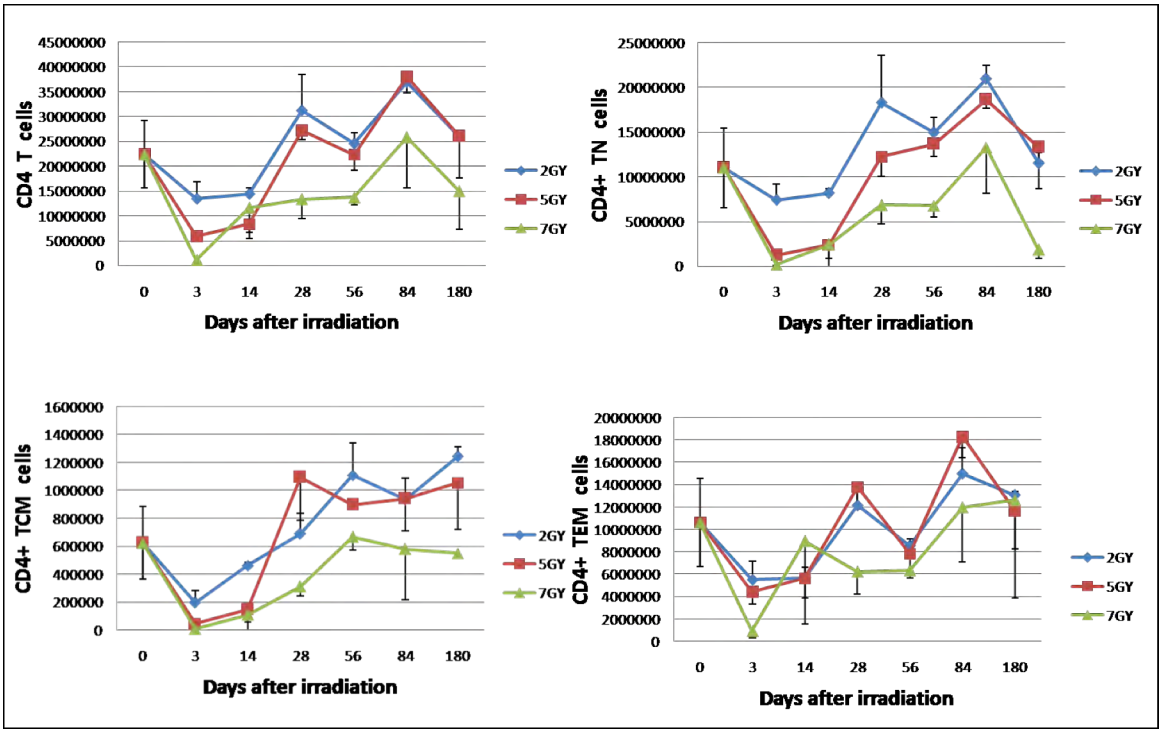
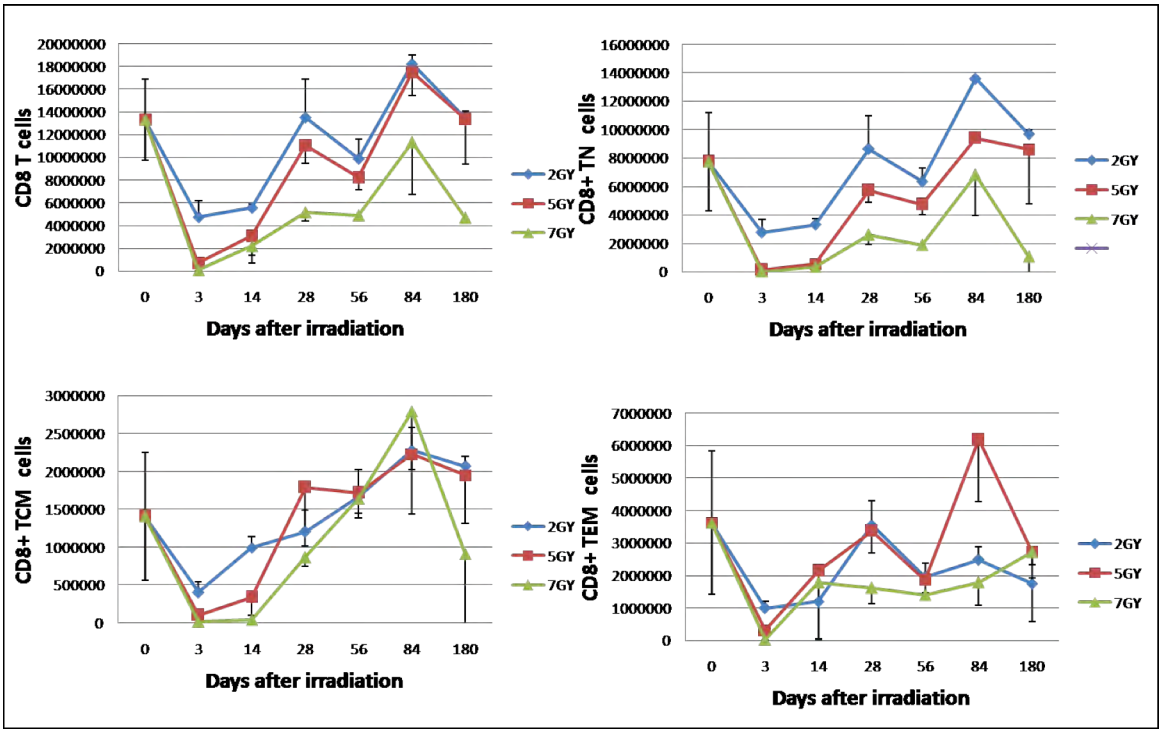


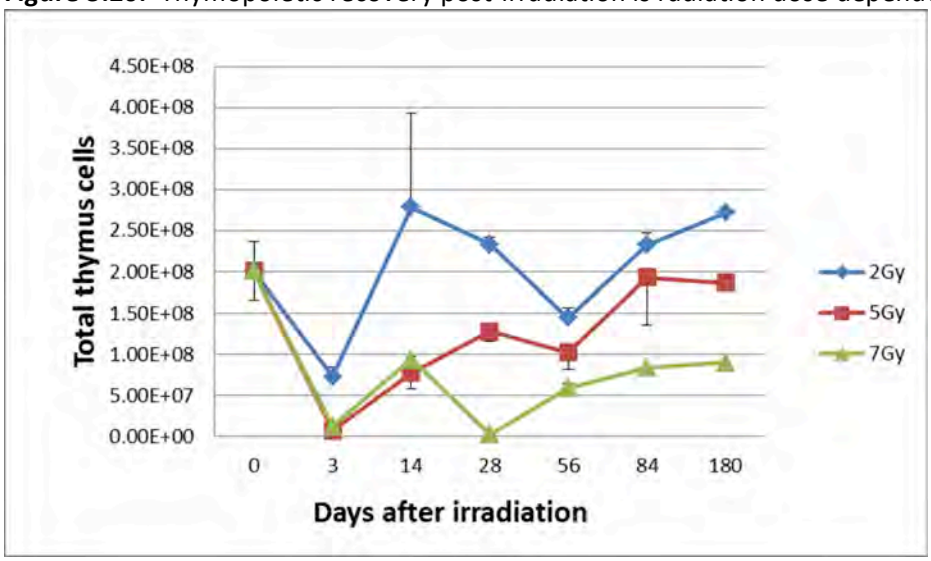
Figure 3.19: CD8 T cell recovery post-irradiation is radiation dose-dependent in mouse spleen



Thymopoietic recovery post-irradiation in mice is radiation dose-dependent:
Total thymocyte recovery was measured at different time post-irradiation. As shown in **Figure 3.20**,

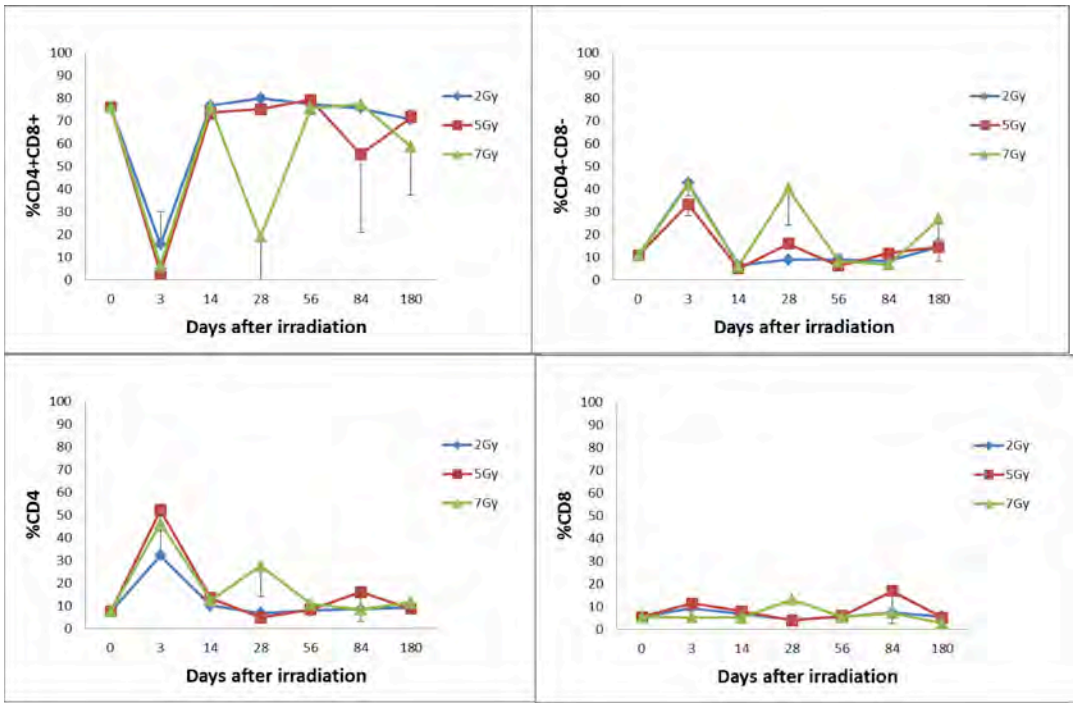
similar to peripheral T cells, total thymocytes dropped rapidly in irradiated mice in a radiation dose-dependent manner at day +3. Thymocytes in the 2 Gy group recovered rapidly, with total thymocyte number recovered to the pre-irradiation level by day +14. It took 84 days for thymocytes to recover to the pre-irradiation levels in the mice irradiated at 5 Gy. Total thymocytes in the 7 Gy group had not recovered to normal when examined at day +180.

Figure 3.20: Thymopoietic recovery post-irradiation is radiation dose dependent



To study the recovery of mouse thymic function, we also monitored the recovery of thymocyte subsets. As shown in **Figure 3.21**, the percentages of double positive subsets recovered backed to normal as soon as 14 days after irradiation in all groups, suggesting that the slow recovery of total thymocytes in higher radiation dose groups is not a result of defective machinery in irradiated thymus. It is of note that the percentage of double positive in the 7 Gy group at day +28 was not be accurate because very low number of thymocytes were analyzed.

Figure 3.21: Thymocyte subset recovery post-irradiation



4) Other Achievements

None

Project 4 - Genomic Sequencing and Stem Cell Lines (Dr. Dave)

There were two main goals of the study. First, to define the genetic mutations and gene expression signatures induced by radiation. Second, define the molecular basis of immune deficiency and hematologic abnormalities induced by radiation.

1) Major activities:
We are developing the parameters for handling high throughput sequencing data and identifying mutations. Next generation sequencing reads in the *fastq* format are being pre-processed with GATK. We are optimizing the parameters for mapping these reads to the to the reference rhesus macaque (Macaca mulatta) genome using Burrows-Wheeler Aligner (BWA). Error rates for different settings are being compared, allowing us to accurately calibrate the performance of this critical step. Base quality calibration and indel realignments will be performed in the next period using GATK to further optimize this process. Once this optimization is complete, we will begin to annotate variants with gene names and predicted function. Once these steps are complete, we will scale-up the process in the upcoming periods.

2) Specific objectives:
Develop calibration/mapping parameters for primate-specific sequencing.

3) Results/key outcomes:
Under aim #1, we developed the methods for whole exome sequencing and transcriptome sequencing from rhesus macaques. Since the methods for the analysis of data from this species are poorly developed, we had to develop these methods. We also sequenced 10 rhesus macaques including five exposed to DEARE. Our early data indicate that we can reliably identify mutations and gene expression from these cases.

In order to define the genetic mutations in rhesus macaques, we had to fully develop the methodologies for next generation sequencing and bioinformatics analysis. In the past year, we developed the methodologies for whole exome sequencing from rhesus macaque DNA.

Briefly, genomic DNA was sheared to 250 bp and size/concentration were verified using Bioanalyzer (Agilent Technologies). Sheared DNA was end-repaired, A-tailed, and ligated to Illumina paired-end adapters. The resulting libraries were amplified using Illumina PE specific primers. Samples were column purified and the size and quantity of the final libraries was determined using Bioanalyzer (Agilent Technologies). The resulting libraries were then hybridized overnight to DNA baits provided in the SureSelect Human All Exon 50MB kit (Agilent Technologies). The captured libraries were amplified and sequenced using the Illumina platform. FASTQs were aligned to the rhesus macaque reference genome using BWA-MEM. Indel realignment and base quality recalibration are done with GATK tools. Variants were called using SAMtools mpileup on all samples. Somatic mutations in paired samples were called using Mutect. Variants were annotated with SNPeff. Average

sequencing depth throughout samples was 80x.

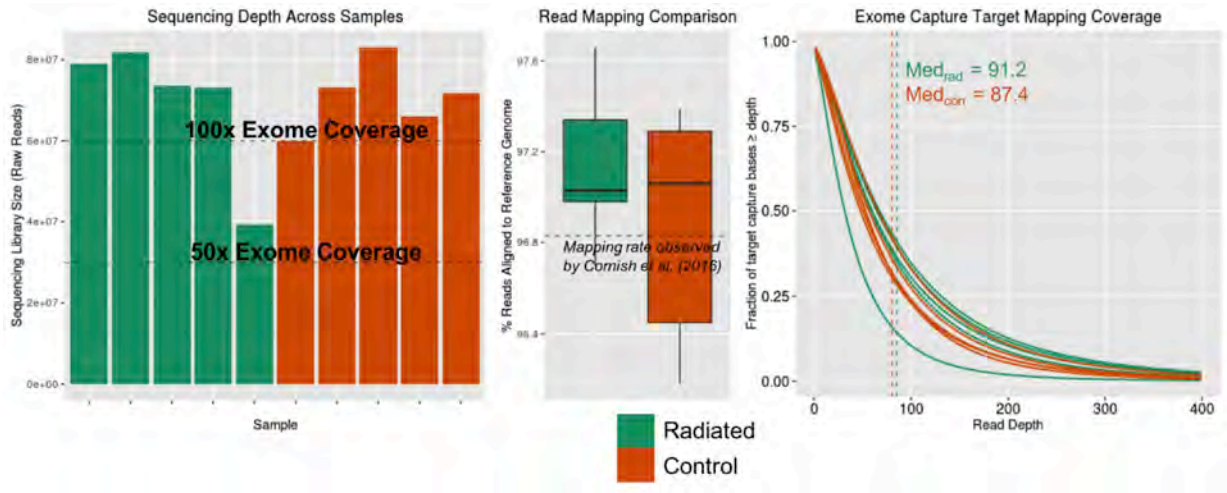


Figure 4.1: Generating whole exome sequencing data from rhesus macaques.

We began by generating sequencing data for five rhesus macauqes that were exposed to radiation and five controls (Figure 4.1). We found that we were able to generate high quality data. The read mapping percentages that we observed were comparable to those seen by Cornish et al (2016).

We further examined the exome capture target mapping percentages for the irradiated and control cases. We found that the mapping percentages were very comparable for both sample types.

These data demonstrate that we can generate high quality sequencing data from rhesus macaques and set up the methodology for our work in the upcoming years of the proposal.

<u>Examination of radiation effects on the evolution of novel genetic variants</u>
We performed an early analysis to examine the potential effects of radiation on the evolution of novel genetic mutations. We therefore analyzed the sequencing data from five irradiated rhesus macaques and five controls and carefully tabulated the different classes of genetic variation including single nucleotide variants, insertions and deletions (Figure 4.2).

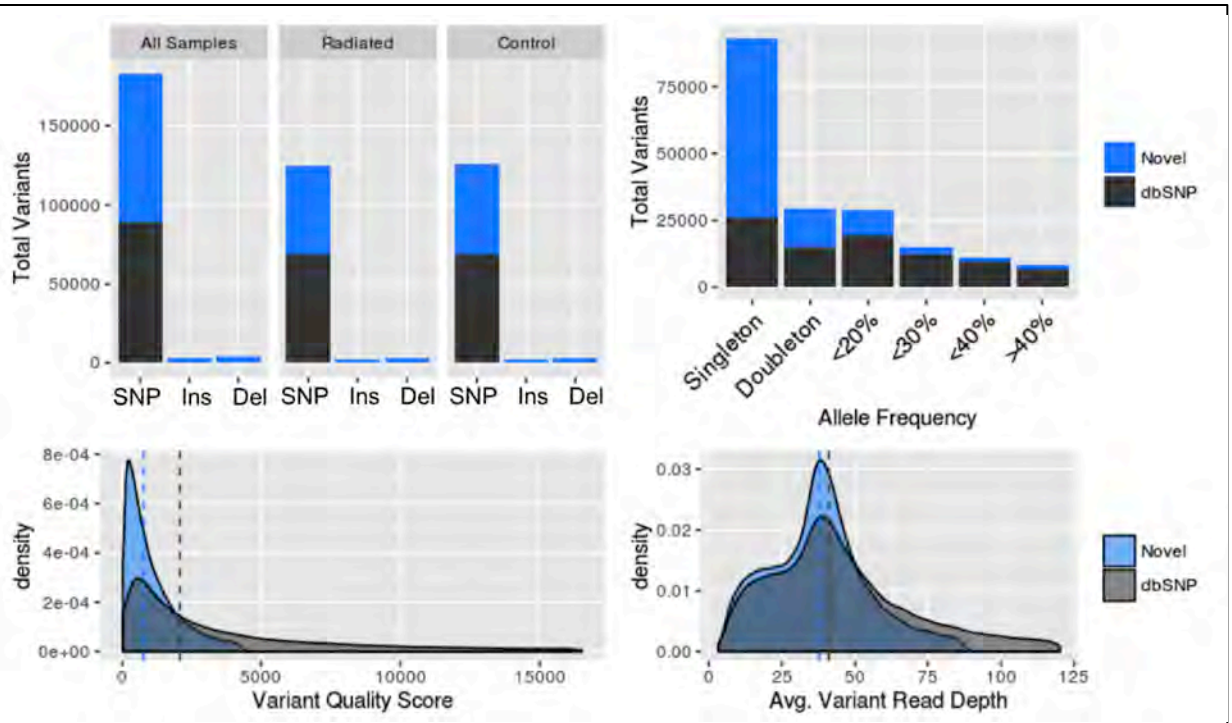


Figure 4.2: Total number of novel variants not influenced by radiation exposure.

In this preliminary analysis we found that the distribution of single nucelotide substitutions, insertions and deletions were very similar for the irradiated and non-irradiated cases. We further examined the number of novel and known genetic variants as a function of sequencing quality as well as read depth. We found that the relative number of known variants in dbSNP and novel variants did not significantly vary at conventional cutoffs used to identify genetic variants.

These early data indicate that irradiation does not dramatically increase the genetic variation measure in vivo after a long interval. The specific nature of the genetic variation remains to be described in the larger sample set planned in the proposal.

Global patterns of genetic variation in radiated cases and controls

We further examined the nucleotide-level genetic variation in the irradiated and control rhesus macaques (Figure 4.3). We examined the genetic alterations using principal component analysis and found that the main principal components did not distinguish irradiated from other cases indicating that irradiation was not the main point of distinction between these cases. We further examined nucleotide-level differences in the irradiated and control cases. We found that, for every possible nucleotide change, the broad characteristics of irradiated and control cases were similar.

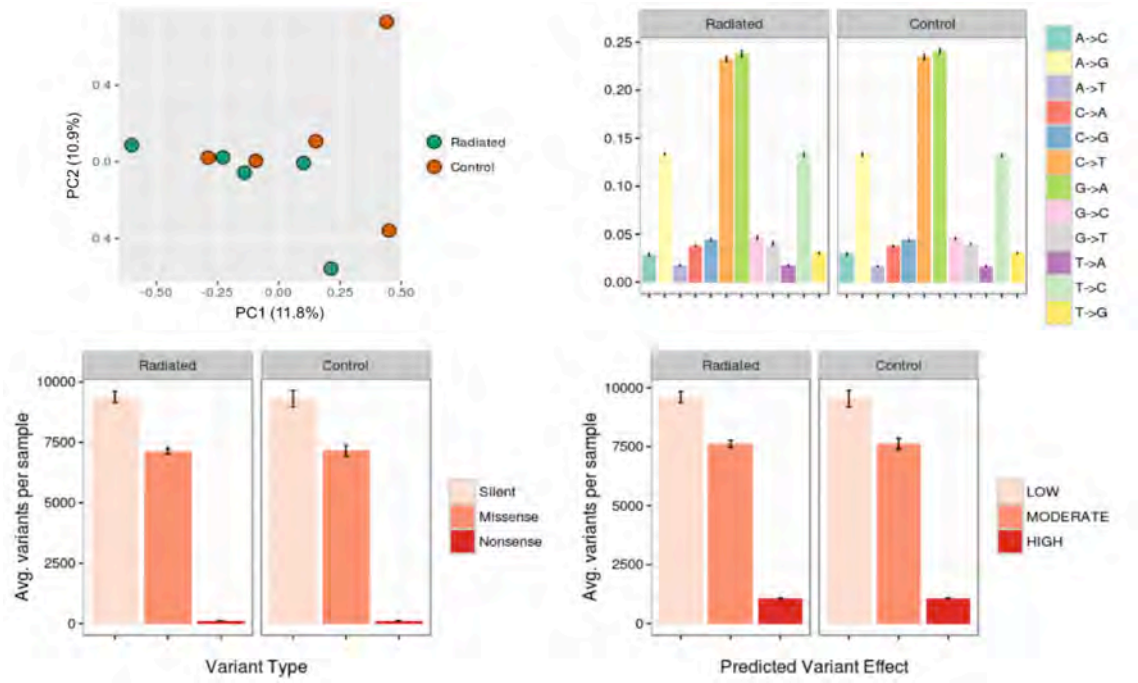
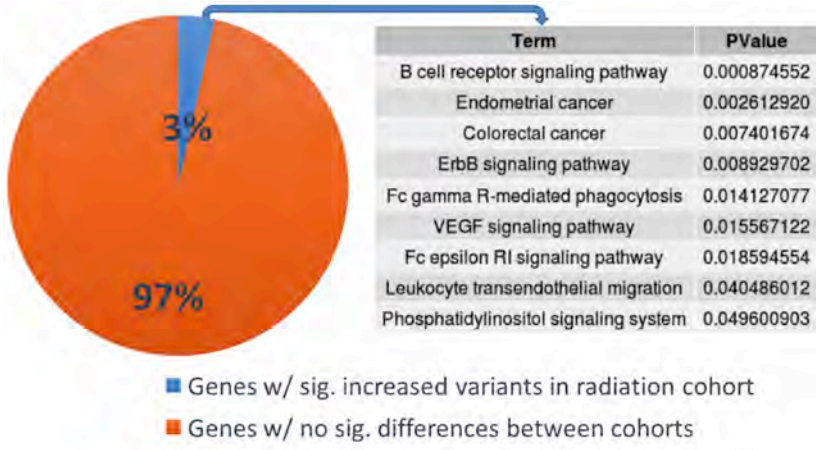


Figure 4.3: Global patterns of genetic variation and radiation exposure.

We further examined the disritbution of synonymous (silent), missense and nosense variants in irradiated versus control samples and found no differences. We also observed general agreement between the two groups in the variants that were predicted to be of low, moderate or high impact based on conservation scores.

<u>Differences in genetic variation between irradiated and control groups.</u>

As expected from the above analysis, we observed very strong overlap between the irradiated and control group with a 97% overlap of genotypes among these groups. To better understand the genetic differences between he two groups, we further examined the pathways that comprise the genetic differences between the two groups (Figure 4.4).



Term	PValue
B cell receptor signaling pathway	0.000874552
Endometrial cancer	0.002612920
Colorectal cancer	0.007401674
ErbB signaling pathway	0.008929702
Fc gamma R-mediated phagocytosis	0.014127077
VEGF signaling pathway	0.015567122
Fc epsilon RI signaling pathway	0.018594554
Leukocyte transendothelial migration	0.040486012
Phosphatidylinositol signaling system	0.049600903

Figure 4.4. Genes with significantly more radiation-associated variants.

We found that B cell receptor signaling pathway was the single most strongly altered pathway, which fits well with our initial hypothesis implicating altered immune functions in irradiated macaques and planned experiments in Aim #2.
Most of the work in this review period focused on Aim 1. Early experiments for Aim #2 are described below.

Develop the a stem-cell based model to generate immune B cells in vitro (AIM #2)

Given the potential multigenic nature of genetic variation and the emerging role for B cell receptor signaling and other immune functions, we proposed to differentiate stem cells from irradiated macaques (or human stem cells with the genetic modifications present in irradiated macaques) into B cells to better understand their function and the long-term effects of radiation on immune function.
We have previously developed the schema shown in Figure 5 below to differentiate B cells from stem cells.

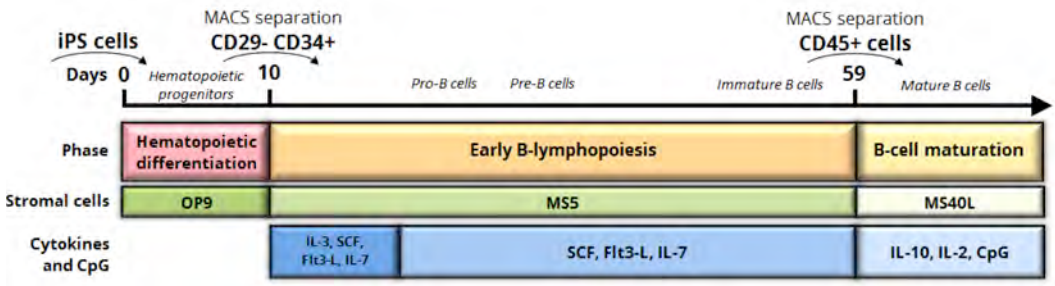


Figure 4.5: Schema for generating B lymphoid cells.

The expected yield of B cells from earlier experiments was roughly 10%. However, the actual yields we observed at 90 days was on-average, less than 1%. These are early experiments, so we are trying to better understand the reasons for the low observed yields. We are repeating these experiments with new reagents.

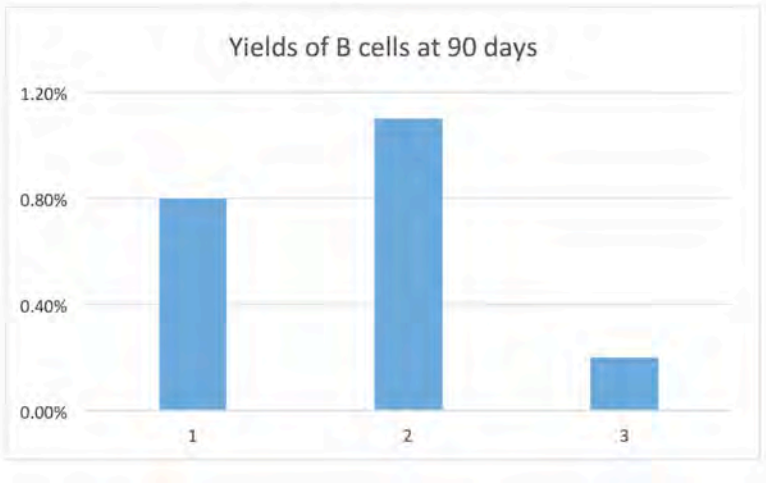


Figure 4.6: Yields of B cells after in vitro differentiation; the Y axis represents B cell

yield; the X axis indicates experimental replicate number.

If we cannot improve the yields of B cells, we have other potential approaches including trying to differentiate the stems cells in vivo using a Nod-Scid-gamma (NSG) mouse model. We will confer with the program officer prior to implementing any changes in experimental approaches, if those are found to be necessary.

4) Other achievements:

None

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Overall (PI Cline) - Nothing to Report; see below by project

Project 1 Diabetes (PI Kavanagh).

1. This project has led to training of the technical staff in data management and analysis.
2. The PI has attended an NIAID scientific update meeting at Duke university which was educational.
3. A new trainee Dr. Katie Fanning has joined the Kavanah lab (July 2016); she will benefit from the training provided by this work but is supported by a separate NIH T32 program.
4. All staff have participated in radiation dosimetry training to enable conduct of the mouse study.
5. The PI has attended an NIAID scientific update meeting at Duke University which was educational as well as disseminating information to the scientific community (1st September 2016).
6. The PI is part of the PhD dissertation committee for the graduate student in Dr. Furdui's laboratory, and whose work relates to redox proteomics relating to Akt2 regulation.

Project 2 RIHD (PI Register)

1. Image Analysis Software, Cardiac Ultrasound, and Cardiac MRI:
2. Tissues: VisioPharm, programmable, working to develop semi-automated programs to assess myocardial phenotypes including fibrosis.
3. Cardiac Ultrasound: CV ultrasound applications expert from GE provided a day long training for investigators and staff, establishing new SOPs for prospective cohort.
4. Cardiac MRI: Register and Michalson had a training session on the use of MIMP software useful for assessments of CMRI images by the developer (Craig Hamilton)
5. Gene expression analyses: Day-long tutorial on the use of Ingenuity Pathways software from applications specialists attended by Drs. Michalson and Register.

Project 3 (Immune Recovery) PI Sempowski and Chen

1. Dr. Ying Huang is a visiting scholar. She has performed work in in vivo radiation studies.
2. Dr. Chen attended 2016 Federation of Clinical Immunology Societies meeting held in June in Boston. This is the meeting in translational immunology delivering the latest breakthroughs across immune-mediated diseases.
3. Dr. Sempowski attended the ThymUS conference in June 2016 in Maui. This meeting is the premier meeting for thymus and immune recovery investigation. Work on the impact of radiation on human thymus was presented by Dr. Sempowski.

Project 4 (Genomics) PI Dave - Nothing to Report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Overall Program (Cline)
Study design, public health and strategic relevance, and opportunities for collaboration were presented by Dr. Cline at several venues:

- The Southeastern Anatomic Pathology Chairs Regional Meeting, Blowing Rock, NC, 9/28/16.
- Wake Forest Department of Pathology Grand Rounds, Winston-Salem, NC, 9/7/16.
- The NIH/NIAID Centers for Medical Countermeasures Against Radiation (Annual meeting 2/25/16, monthly teleconferences with the CMCR Steering Committee)
- Invited speaker at an FDA Forum "Exploring Late Effects of Radiation Exposure" - FDA, Silver Springs, MD, 2/25/16
- Comparative Medicine Research Strategy Meetings
- Wake Forest Animal Resources Program Continuing Education Seminar

Project 1 - Diabetes (Kavanagh)

- Attended an NIAID scientific update meeting at Duke University which was educational as well as disseminating information to the scientific community (1st September 2016).

Project 2 - Radiation-Induced Heart Disease (Register)

- Attended the aforementioned NIAID scientific update meeting at Duke University (1st September 2016).

Project 3 - (Immune Recovery) (Semposki/Chen)

- Attended the aforementioned NIAID scientific update meeting at Duke University (1st September 2016).

Project 4 - Genomics (Dave)

- Attended the aforementioned NIAID scientific update meeting at Duke University (1st September 2016).

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Overall (PI Cline): No Change. For all projects we anticipate continued local and national presentations of results at relevant scientific meetings, and publications of results in the peer-reviewed literature.

Project 1 (Diabetes, PI Kavanagh): No Change

Project 2 (RIHD, PI Register): No Change. We plan to continue to collect samples from the prospective and long term cohorts, to continue to analyze echocardiographic and cardiac MRI datasets, to complete paperwork for transfer of samples between Denmark and Make Forest , and to work with Duke investigators in plans for gene expression analyses in blood.

Project 3 (Immune Recovery, PI Sempowski/Chen): Presentations, abstracts, meetings and manuscripts as studies mature

Project 4 (Genomics, PI Dave): Abstracts/meetings and manuscripts as studies mature. In the coming year, we will scale up the sequencing. We will sequence 30 whole exomes and 30 transcriptomes to preliminarily define the genomic signatures associated with DEARE. For AIM #2, we will repeat our experiments to generate B cells from stem cells. We will increase the exposure to CD40L, which is known to stimulate B cell production and differentiation. We will carefully monitor these experiments for increased efficiency of lymphoid differentiation.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project.

Summarize using language that an intelligent lay audience can understand (Scientific American style).

The findings produced by this program have increased awareness of the multi-systemic nature of late radiation effects, in the domains of radiation biology, radiation mitigation, and medical management of affected individuals post-exposure.
Our data have shown new findings such as: Dysregulation of systemic metabolic function, leading to diabetes mellitus; chronic persistent whole-body increases in circulating inflammatory signals, years after exposure; diffuse myocardial injury leading to diastolic dysfunction (heart failure with preserved ejection fraction) and fibrosis; and long-term immune impairment. Future elucidation of the underlying genomic, transcriptomic and functional consequences is anticipated as Project 4 (Genomics/Dave) progresses into the analytic phase in the coming year.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

This impact of our findings is broad, and spans multiple groups and agencies working to understand and mitigate delayed effects of acute radiation exposure, including the DOD, BARDA, NIH, FDA, and NASA. We anticipate that our data will influence response strategies, biomarker development, and re-adjustment of regulatory assumptions regarding the modeling, prediction and treatment of the long-term consequences described herein.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:
- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. CHANGES/PROBLEMS: The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

We have made no substantial changes in any Project during Year 1 of this Program.

Minor changes are listed below:

Overall: No substantive changes in approach. The radiation dose given to the prospective NHP cohort was adjusted downward from 6 Gy to 4 Gy, in order to reduce the chance of mortality. This minor change is consistent with recently published literature on the measured radiation sensitivity of rhesus macaques.

Project 1 (Diabetes, PI Kavanagh): No substantive changes in approach have occurred.
We have reduced our radiation dose planned for the mouse study (Aim 4) from 8 to 7 Gy due to concerns about lethality. We are also comparing methodologies for measuring A1c values to find a more reliable laboratory for ongoing use.
We have reduced our radiation dose planned for the mouse study (Aim 4) from 8 to 7 Gy due to concerns about lethality. A proof of concept study has been completed (data shown above) to substantiate that this reduced dose of radiation results in acceptable survivability and induces reliable decreases in glucose metabolism. We plan to request approval from the agency to change our intervention strategy to more directly target the pathological changes observed in monkeys.

At the time of grant preparation, we proposed to intervene in our study of total body irradiation with a combination of agents that would restore adiposity and reduce fibrosis. These decisions were based on available data from irradiated non-human primates (NHPs) which had insulin resistant skeletal muscle even when overt diabetes (DM) was not present. Since then, we have delved further into the NHP model to demonstrate that in the face of increased extracellular matrix accumulation post-irradiation (data shown above), that skeletal muscle capillary density and associated nitric oxide metabolites differentiated those who went on to develop DM (data shown above). Thus, compensation of skeletal muscle to increase effective myocyte surface area (and thus capacity for the majority of glucose disposal by increasing perfusion) looks to be a potentially protective strategy. For this reason, we believe that testing this hypothesis in the mouse model is the logical next step. We have piloted our mouse model and have demonstrable insulin resistance post-irradiation (data shown above). In our proposed experiment planned for imminent start, we would request permission to substitute therapy with a thiazolidinedione (aimed to modify fat deposition and with known insulin sensitizing action) with tadalafil. Tadalafil is a phosphodiesterase 5 inhibitor ([PDE5] prescribed as Cialis), which recent accumulation of evidence demonstrates positive effects on compromised skeletal muscle blood flow. Tadalafil (and compounds of this same class) are currently readily available, cheap, and safe making them a viable option for the treatment of DM as a delayed effect of radiation exposure.

PDE5 inhibition has been shown to restore exercise-activated perfusion in aged muscle through improved nitric oxide signaling, and improves perfusion in clinical cases of muscular dystrophy. We hypothesize that this agent alone will be able to

improve insulin signaling related to measures of perfusion but also may reduce extracellular matrix deposition as has been seen in models of DM renal disease. Thus in our 2 x 2 factorial study design, we expect to see additive or synergism with blockade of the fibrosis development. We propose to dose mice with 5mg/kg/d tadalafil based on prior reports. This dose is higher than used in these reports but accounts for the waste associated with dosing via compounding in the food versus daily oral gavage. This dose is expected to approximate 20mg/day administered to people.

Currently we have proposed to use angiotensin receptor blockade to reduce fibrosis. We now know that the master signal for fibrosis, TGFß, is a druggable target. TGFβ antagonists have demonstrated safety and early signs of efficacy. There are TGFβ antagonists currently in clinical trials for a variety of diseases, including the small molecule inhibitor of TGFβRI, galunisertib. IPW-5371 (Innovation Pathways Inc.), is a small molecule inhibitor of TGFβRI kinase, with similar potency to galunisertib, a superior pharmacokinetic profile in mice, and the potential for an improved therapeutic index. The structural class of TGFβRI inhibitors represented by IPW-5371 have a prolonged circulating half-life when tested in vivo. In contrast, the TGFβ inhibitors developed by Eli Lilly, including galunisertib, are rapidly cleared, requiring twice daily dosing, and have a high peak/trough ratio that could lead to undesired toxicities. IPW-5371 is a chemically stable, orally bio-available, potent, inhibitor of TGFβRI with an excellent pharmacokinetic profile can mitigate the delayed effects of thoracic radiation at 10 and 30 mg/kg doses. IPW-5371 is effectively inhibits TGFß signaling, fibrosis markers (hydroxyproline), improves organ function (lung and heart) and extends survival in a thoracic radiation mouse model. These results have been recently published (Rabender et al. Rad Res 2016; 186: 478-488) and the company has discussed with us use of their compound for DM prevention and agreed to provide the amount required to perform the planned mouse study in Year 2 of this project.

We are also comparing methodologies for measuring A1c values to find a more reliable laboratory for ongoing use. Primate samples have shown a tendency to read low values with immunoassay based methods are used. We have preferred to use chromatographic methods for this outcome and we are testing other laboratories and a handheld device for their accuracy.

Project 2 (RIHD, PI Register): Nothing to Report.

Project 3 (Immune Recovery, PI Sempowski/Chen): Nothing to Report.

Project 4 (Genomic, PI Dave): Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Overall: We encountered minor difficulty in scheduling NHP irradiation procedures in September 2016 as originally planned; these procedures were moved to October 2016. We do not anticipate any adverse effect of this minor 1-month delay.

Project 1 (Diabetes, PI Kavanagh): The start of SA4 (prevention of DM development in irradiated mice) has been delayed from the original October plan. We plan to acquire mice in January. The delay has resulted from the need for protocol amendment, desire to change interventional agents to be more specific in targeting muscle pathology, and the need to leave enough time for required approvals We anticipate that these minor delays will not impede the timely completion of the planned work.

Project 2 (Radiation Induced Heart Disease, PI Register) - Nothing to Report.

Project 3 (Immune Recovery, PI Sempowski/Chen) - Nothing to Report.

Project 4 (Genomic, PI Dave) - Nothing to Report.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Overall: As noted in a prior quarterly progress report, the cost of purchasing 20 NHPs for the prospective studies was higher than anticipated ($6,500 rather than $5,000). This unexpected and unavoidable cost increase was offset by savings due to delays in hiring technical staff and by the use of other sources of funding (NIH T32 Training Program) for support of post-doctoral fellows.

Project 1 (Diabetes, PI Kavanagh): Nothing to report.

Project 2 (Radiation-Induced Heart Disease, PI Register): Nothing to report.

Project 3 (Immune Recovery, PI Sempowski/Chen): Nothing to report.

Project 4 (Genomics, PI Dave): Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not applicable - No human subjects

Significant changes in use or care of vertebrate animals.

Overall (PI Cline; All Projects)

In our overarching cross-project Nonhuman Primate Protocols - A variety of minor protocol amendments were made throughout the year, reflecting personnel changes, minor procedural changes, and administrative changes (e.g. protocol renewal).

Core Primate Studies (Cline)

PROTOCOL 1 of 4 total:

Protocol [ACURO Assigned Number]: PR141508.04

Title: **Radiation Countermeasures Long Term Response of Rhesus Macaques**

Target required for statistical significance: **120**

Target approved for statistical significance: **132**

Submitted to and Approved by:

Wake Forest IACUC (9/17/13; WF IACUC no. A13-117; Renewed/reapproved 6/23/16 as A16-094 as part of routine full renewal every 3 years as per IACUC policy)

Amendments since our prior quarterly report are shown below.

Protocol # A16-094 - Radiation Survivor Core

Amendment No.	Reason for amendment	Wake Forest IACUC approval date	ACURO approval date
A16-094	Replacement of A13-117	6/23/16	7/25/16
1	Personnel; addition of new employees	6/29/16	7/25/16
2	Addition of adenosine infusion	8/1/16	8/4/16
3	Personnel; addition of new employee; removal of employees	8/3/16	8/4/16
4	Addition of rectal collection of fecal material	8/8/16	8/12/16
5	Personnel; addition of new employees	8/11/16	8/12/16

PROTOCOL 2 of 4 total:

Protocol [ACURO Assigned Number]: PR141508.01

Title: **Prospective Radiation Cohort: Cardiac and Metabolic Studies**

Target required for statistical significance: 20

Target approved for statistical significance: 28

Submitted To And Approved By:

Wake Forest IACUC (Protocol #A15-083, approved 7/6/15)

ACURO approval (9/3/15, ACURO no. PR141508.01)

Amendments since last quarterly progress report.

Amendment No.	Reason for amendment	Wake Forest IACUC approval date	ACURO approval date
8	Personnel; addition of new employee	7/6/16	7/7/16
9	Personnel; addition of new employee	7/8/16	7/9/16
10	Personnel; addition of new employee	7/11/16 (withdrawn)	NA
11	Addition of adenosine infusion	7/28/16	8/4/16
12	Personnel; addition of new employee; removal of employees	8/2/16	8/4/16
13	Personnel; addition of new employee; removal of employee	8/3/16	8/4/16
14	Addition of rectal collection of fecal material	8/8/16	8/11/16
15	Personnel; addition of new employee	8/30/16	pending
16	Addition of bone marrow collection	9/14/16	pending

STATUS:

Animals are undergoing irradiation and acute supportive care.

PROTOCOL 3 of 4 total:

Protocol: Wake Forest IACUC #A15-068; ACURO Assigned Number PR141508.02
Title: **Prospective Evaluation of Diabetogenesis Post-irradiation in Mice (Metabolic Effects of Radiation Exposure)**
Target required for statistical significance: 200

Target approved for statistical significance: 200

Submitted To And Approved By:

Wake Forest IACUC (A15-068, Approved 5/20/15

ACURO (PR141508.02, Approved 10/19/15)

Amendments:

Amendment No.	Reason for amendment	Wake Forest IACUC approval date	ACURO approval date
1	Addition of non-invasive spectroscopy	6/25/15	N/A - incorporated prior to initial ACURO review
2	Personnel change	7/9/15	N/A - incorporated prior to initial ACURO review
3	ACURO request to change USDA category to type D	8/26/15	N/A - incorporated during initial ACURO review
4	Personnel change	7/11/16	pending

Status:

Animals have not yet been acquired.

PROTOCOL 4 of 4 total:

Protocol [ACURO Assigned Number]: PR141508.03
Title: IMMUNOLOGICAL INJURY AND RECOVERY AFTER RADIATION INJURY
Target required for statistical significance: 4184 mice
Target approved for statistical significance: 4184 mice

SUBMITTED TO AND APPROVED BY:

Duke IACUC (A142-15-05, Approved on 5/28/15)

ACURO (PR141508.03, Approved on 3/3/16)

No additional amendments since last quarterly progress report.

Status - Approved, work in progress.

Significant changes in use of biohazards and/or select agents

No Significant Changes

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Overall (Cline): Nothing to Report.

Project 1 (Diabetes, PI Kavanagh): Nothing to Report.

Project 2 (Radiation-Induced Heart Disease, PI Register): One publication.

DeBo RJ, Lees CJ, Dugan GO, Caudell DL, Michalson KT, Hanbury DB, Kavanagh K, Cline JM, Register TC. Late Effects of Total-Body Gamma Irradiation on Cardiac Structure and Function in Male Rhesus Macaques. Radiat Res. 2016;186:55-64. doi: 10.1667/RR14357.1. Epub 2016 Jun 22. PubMed PMID:27333082.
(http://www.ncbi.nlm.nih.gov/pubmed/27333082)
Federal Support was acknowledged.

Project 3 (Immune Recovery, PI Sempowski/Chen): Nothing to Report.

Project 4 (Genomics, PI Dave): Nothing to Report.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

ABSTRACTS

Overall (Cline): One abstract (invited presentation).

J Mark Cline1; Greg Dugan1; Rachel Andrews1; Kylie Kavanagh1; Daniel Bourland1; David Hanbury1; Ann Peiffer1; Thomas Register1; Ryne DeBo1; David Caudell1; and Nelson Chao2
Multisystemic late effects of radiation exposure in nonhuman primates.
Wake Forest University School of Medicine, Winston-Salem, NC1 and Duke University Medical Center, Durham, NC2
Abstract accepted; Radiation Research Society Annual Meeting 2016
Federal support acknowledged.

Project 1 (Diabetes PI Kavanagh): Two abstracts.

1. B.R. Pfisterer.1, 2, A.T. Davis 2, T.D. Presley3, D. Wasserman4, J.M. Cline2, K. Kavanagh2, 1Cummings School of Veterinary Medicine, Tufts University, North Grafton, MA; 2Department of Pathology, Wake Forest School of Medicine , Winston Salem, NC; 3Department of Chemistry, Winston Salem State University, Winston Salem, NC 4Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN
The long term effects of ionizing radiation on muscle extracellular matrix and perfusion
Abstract accepted; American College of Veterinary Pathologist Annual Meeting 2015
Federal support acknowledged.

2. K. Kavanagh1, B.R. Pfisterer1, A.T. Davis 1, T.D. Presley2, I.M. Williams3, D. Wasserman3, J.M. Cline1
1Department of Pathology, Wake Forest School of Medicine , Winston Salem, NC; 2Department of Chemistry, Winston Salem State University, Winston Salem, NC 3Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN
The long term effects of ionizing radiation on muscle extracellular matrix and perfusion in monkeys
Abstract accepted; Radiation Research Society Annual Meeting 2016
Federal support acknowledged.

Project 2 (RIHD, PI Register): One abstract.

Abstract accepted for presentation at 2016 Annual Radiation Research Meeting: (see Appendix).
Register TC; Debo R; Michalson K, Lees CJ, Dugan G, Sempowski G, Kavanagh K, Caudell D, and Cline JM. Serum IL-10 and IL-15 Levels are Increased with Prior
Exposure to gamma irradiation is associated with radiation sensitive cardiac phenotypes
Federal funding from NIAID and DOD was acknowledged.

Project 3 (Immune Recovery, PI Sempowski/Chen): Nothing to Report.

Project 4 (Genomics, PI Dave): Nothing to Report.

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Overall: (PI Cline) - Nothing additional to report.
Project 1 (Diabetes, PI Kavanagh): Nothing to report.
Project 2 (Radiation-Induced Heart Disease, PI Register): Nothing to report.
Project 3 (Immune Recovery, PI Sempowski/Chen): Nothing to report.
Project 4 (Genomics, PI Dave): Nothing to report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for

any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*

Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award).*

Core Primate Studies (Cline)

Name:	J. Mark Cline *No change from previous submission*
Name:	Daniel Bourland *No change from previous submission*
Name:	David Caudell *No change from previous submission*
Name:	Janet Tooze *No change from previous submission*
Name:	Jean Gardin *No change from previous submission*
Name:	Matt Dwyer *No change from previous submission*
Name:	Russell O'Donnell *No change from previous submission*
Name:	Chrystal Bragg *No change from previous submission*
Name:	Renae Hall *No change from previous submission*
Name:	Patricia Warren *No change from previous submission*

Project 1 – Diabetes (Kavanagh)

Name:	Kylie Kavanagh *No change from previous submission*
Name:	Ashley Davis *No change from previous submission*
Name:	Christina Sherrill *No change from previous submission*
Name:	Cristina Furdui *No change from previous submission*
Name:	Xiaofei Chen *No change from previous submission*

Project 2 – Radiation/Heart Disease (Register)

Name:	Thomas Register *No change from previous submission*
Name:	Sujethra Vasu
Project Role:	Co-Investigator
Researcher Identifier:	N/A
Nearest person month worked:	1.2 (effort started 07/01/2016)
Contribution to Project:	Dr. Vasu was budgeted on the original submission and effort has only recently started. Dr. Vasu is a cardiologist working with Drs. Hamilton and Register in the conduct and analysis of cardiac MRI studies.
Name:	Craig Hamilton
Project Role:	Co-Investigator
Researcher Identifier:	N/A
Nearest person month worked:	0.6 (effort began 07/01/2016)
Contribution to Project:	Dr. Hamilton is an addition to the project and he brings expertise in the area of analysis of the cardiac MRI studies. He will work with Drs. Register and Vasu to interpret these studies.
Name:	Kris Michalson, DVM
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Dr. Michalson performed statistical analysis, writing, and revision of the manuscript: Late Effects of Whole Body Gamma Irradiation on Cardiac Structure and Function in Male Rhesus Macaques which is now in press in Radiation Research Conducting background research regarding radiation induced heart disease: focusing on radiation induced cardiac fibrosis, specifically the contributions of cardiac fibroblasts, circulating monocytes, tissue macrophages, and the differences in endocrine and paracrine signaling pathways and biomarkers in acute and chronic fibrosis promotion. Received training in echocardiography using the GE Logiq S8, Visiopharm Image Analysis software, Olympus VS120 slide scanner, and the use of specially designed software to analyze cardiac MRI images. Attended a daylong genomics courses in the use of INGENUITY software and another in the use of data from the ENCODE project. Analyzed the echocardiography studies for the baseline period of the prospective cohort.

Name:	James Bottoms ***No change from previous submission***
Name:	Maryanne Post
Project Role:	Laboratory Technician IV
Researcher Identifier:	N/A
Nearest person month worked:	1.2 (effort began 07/01/2016)
Contribution to Project:	**Maryanne was budgeted in the original submission and effort has recently started. Ms. Post is an experienced laboratory technician who will perform enzyme assays, radioimmunoassays, histology, and other laboratory bench work in support of this project**.

Project 3 – Immune Recovery (Duke Consortium – Chen and Sempowski)

Name:	Benny Chen
Project Role:	PD/PI
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2
Contribution to Project:	No change
Name:	Gregory Sempowski
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	No change
Name:	Melissa Samo
Project Role:	Senior Laboratory Analyst
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 (left Duke May 31)
Contribution to Project:	Left Duke May 31, 2016, replaced by Dr. Andrew Macintyre and Dr. Laura Hale
Name:	Lesslee Arwood
Project Role:	Research Technician II
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2.5
Contribution to Project:	No change
Name:	Yiqun Jiao
Project Role:	Senior Research Associate
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	No change
Name:	Ying Huang
Project Role:	Visiting Scholar
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	4
Contribution to Project:	No change
Name:	Laura Hale, MD, PhD
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Added June 2016 (10%). Dr. Hale is an immunologist and board-certified pathologist. She is a world expert in thymus morphology and function and will specifically work with Drs. Sempowski and Chen to

	design mouse and NHP studies/analyses to accomplish the 2 aims of Project 3.
Name:	Andrew Macintyre, PhD
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Added Sept 2016 (10% effort). Dr. Macintryre is an immunologist with expertise in peripheral T cell biology, flow cytometric analysis, immune recovery, and immune function. He will be Project lead for all NHP studies and coordinate the Sempowski lab staff to accomplish the 2 aims of Project 3.

Project 4 - Genomic Sequencing and Stem Cell Lines (Duke Consortium – Dave)

Name:	Sandeep Dave
Project Role:	Project 4 PI
Nearest person month worked:	1.2
Contribution to Project:	No change

Name:	Anupama Reddy
Project Role:	Bioinformatics specialist
Nearest person month worked:	3
Contribution to Project:	No change

Name:	Deepthi Rajagopalan
Project Role:	Research Associate
Nearest person month worked:	6 (left Duke August 2016)
Contribution to Project:	Assisted with in vitro experiments and sample processing

Name:	Tiffany Tzeng
Project Role:	Research Technician II
Nearest person month worked:	6 (left Duke August 2016)
Contribution to Project:	Ms. Tzeng worked on iPS cells and validation.

Name:	Eileen Smith
Project Role:	Research Technician II
Nearest person month worked:	2 (joined Duke August 2016)
Contribution to Project:	Assisted with in vitro experiments and sample processing

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

J. Mark Cline (PI)

Changes in *Current* Support as reported in the original application:

Project Title: CD40/PET Pilot Study (Pfizer)
Change: Project complete

Project Title: Summer Veterinary Student Research Fellows at Wake Forest University (T35 - OD 010946)
Change: Transferred to new PI (K. Kavanagh)

Project Title: Center for Medical Countermeasure Against Radiation (Core D – U19 AI67798)
Change: Renewed (see below)

Project Title: Center for Medical Countermeasure Against Radiation (Survivor Core – U19AI67798)
Change: Renewed (see below)

Project Title: Center for Medical Countermeasure Against Radiation (Supplement – U19AI67798)
Change: Project complete

Project Title: Vervet Research Colony as a Biomedical Resource (P40 OD010965)
Change: Project complete

Project Title: Oxygen, Gastrin-Releasing Peptide & Radiation-induced Pulmonary Fibrosis
Change: New Award

Daniel Bourland (Co-Investigator)

Changes in *Current* Support as reported in the original application:

Project Title: Training Program In Translational Radiation Oncology (T32CA11367)
Change: Project Complete

Project Title: Center for Medical Countermeasure Against Radiation (U19 AI67798-06)
Change: Project Complete

Project Title: Center for Medical Countermeasure Against Radiation (Supplement – U19 AI67798-01)
Change: Project Complete

Project Title: Center for Medical Countermeasure Against Radiation (Survivor Core – U19AI67798)
Change: Another five year grant cycle funded (08/01/15 – 07/31/20)

Project Title: Modulation of Radiation-induced Brian Injury in the Nonhuman Primate (1R01CA155293-01)
Change: Project Complete

Project Title: Planimetry for Assessment of Vessicant Chemical-induced Skin Injury (subcontract GTS 44511)
Change: New Award

Project Title: Biodosimetry after Radiological and Nuclear Events (subcontract GTS 43942)
Change: New Award

Changes in *Pending* Support as reported in the original application:

Project Title: Center for Medical Countermeasure Against Radiation (Core D - U19AI67798)
Change: Another five year grant cycle funded (08/01/15 – 07/31/20)

David Caudell (Co-Investigator)

Changes in *Current* Support as reported in the original application:

Project Title: MicroRNA Expression in the NZB/W Lupus Mouse (R15AR062883)
Change: Project Complete

Project Title: Center for Medical Countermeasure Against Radiation (Core D – U19 AI67798)
Change: Renewed (see below)

Project Title: Center for Medical Countermeasure Against Radiation (Survivor Core – U19AI67798)
Change: Renewed (see below)

Project Title: Dmp1-p53/YY1 Interplays and Their Roles in Lung Cancer Development
Change: Project Complete

Project Title: Development of Radiation Dosage Parameters for Lymphodepletion with Minimal Myeloablation in Cynomolgus Maraques
Change: New Award

Christina Furdui (Co-Investigator)

Changes in *Current* Support as reported in the original application:

Project Title: Analysis of Redox Modulated Signaling Networks in Response to Ionizing Radiation (R01 CA136810)
Change: Project Complete

Project Title: Structures and Redox Chemistry in Sulfinic Acid Reduction (R01 GM072866)
Change: Project Complete

Project Title: NFκB and Chromatin Changes in Human Sepsis (R01 AI065791)
Change: Project Complete

Project Title: Structure and functional studies of Akt2 oxidation (U01296)
Change: Project Complete

Project Title: Dietary Intervention to Reduce Breast Cancer Risk: Monitoring Epigenetic Change and Protein Expression Center for Integrative Medicine
Change: Project Complete

Project Title: Regulation of Urinary Bladder Carcinogensis and Progression by SPARC (R01 CA193437)
Change: New Award

Project Title: A Systematic Approach to Evaluate the Infectious Etiology of Breast Cancer (BC15135)
Change: New Award

Project Title: 2016 Thiol-based Redox Regulation & Signaling GRC and GRS (R13 AG053038)
Change: New Award

Kylie Kavanagh (Co-Investigator)

Changes in *Current* Support as reported in the original application:

Project Title: Chaperone Proteins in a Primate Model of Age-related Metabolic Disease (K01 AG033641)
Change: Project Complete

Project Title: Restoring Tissue HSP90 Levels Post-irradiation to Prevent T1DM as a Delayed Effect of Radiation
Change: New Award

Thomas Register (Co-Investigator)

Changes in *Current* Support as reported in the original application:

Project Title: Epigenetics of Diet and Menopause in Nonhuman Primates (subcontract 1U24 DK097748)
Change: Project complete

Project Title: Center for Medical Countermeasure against Radiation (Survivor Core – U19 AI67798)
Change: Renewed (see below)

Project Title: Analysis of plasma Apo A1 and Apo B levels (LipimetriX Private Sector)
Change: Project Complete

Project Title: Holding Protocol for Diet-Induced Hypercholesterolemia in Cynomolgus Monkeys (LipimetriX Private Sector)
Change: Project Complete

Project Title: Evaluation of the Efficacy and Safety of Apolipoprotein Mimetic Peptides (LipimetriX Private Sector)
Change: Project Complete

Janet Tooze (Co-Investigator)

Changes in *Current* Support as reported in the original application:

Project Title: Resonance Imaging Trial for Heart Biomarkers (RITHM) in AYA Cancer Survivors
Change: Project Complete

Project Title: Visceral Adipose Tissue as Mediator between Lifestyle Factors and Cardiometabolic Risk Factors
Change: Project Complete

Project Title: Dietary Protein and Change in Physical Function in Black and White Older Adults
Change: Project Complete

Project Title: Enhancing ARIC Infrastructure to Yield a New Cancer Epidemiology Cohort
Change: Project Complete

Project Title: Minimizing Physical Function Decline in Older Adults Receiving Chemotherapy
Change: Project Complete

Project Title: Effect of Muscadine Grape Extract on Breast and Prostate Cancer
Change: New Award

Project Title: Untangling Sodium, Energy, and Blood Pressure in Whites and African Americans
Change: New Award

Janet Tooze (Co-Investigator) - *continued*

Project Title: Trial of Vitamin D Supplementation and Neuromuscular Function in Older Adults
Change: New Award

Project Title: SEARCH Nutrition Ancillary Study (SNAS2)
Change: New Award

Project Title: Comprehensive Cancer Center of Wake Forest University – Cancer Center Support Grant
Change: New Award

Project Title: Molecular Detection of DNA Hydroxymethylation for Cancer Screening
Change: New Award

Project Title: A Stepped-Care Telehealth Approach to Treat Distress in Rural Cancer Survivors
Change: New Award

Project Title: Prepare to Care: A Supported Self-Management Intervention for Head and Neck Cancer Caregivers
Change: New Award

Project Title: Mixed Methods Examination of Diverse Cancer Patient's Precision Oncology
Change: New Award

Project Title: Development of a Total Nutrient Index
Change: New Award

Sempowski (Duke)

No changes to report

Benny Chen (Duke)

No changes to report

Sandeep Dave (Duke)

Project Title: 5P30-CA14236-40: Cancer Center Program Leadership
Change: New appointment

Project Title: R01CA193655-01: Defining the functional role of mutations in diffuse large B cell lymphoma
Change: New award

Project Title: Leukemia and Lymphoma Society Study 6492-16.
Change: Project ended.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."
Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*

- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

Not Applicable

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

See attachment 1.

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Attachments:

1. Updated Quad Chart

2. Manuscript

   DeBo RJ, Lees CJ, Dugan GO, Caudell DL, Michalson KT, Hanbury DB, Kavanagh K, Cline JM, Register TC. Late Effects of Total-Body Gamma Irradiation on Cardiac Structure and Function in Male Rhesus Macaques. Radiat Res. 2016;186:55-64. doi: 10.1667/RR14357.1. Epub 2016 Jun 22. PubMed PMID:27333082.
   Federal support acknowledged.

3. Abstract

Cline JM[1], Dugan G[1], Andrews R[1], Kavanagh K[1], Bourland D[1], Hanbury D[1], Peiffer A[1], Register T[1], Debo R[1], Caudell D[1], Chao N[2].
Multisystemic late effects of radiation exposure in nonhuman primates
[1]Wake Forest University School of Medicine, Winston-Salem, NC; and [2]Duke University Medical Center, Durham, NC
Abstract accepted; Radiation Research Society Annual Meeting 2016
Federal support acknowledged.

4. Abstract

Pfisterer BR[1, 2], Davis AT [2], Presley TD[3], Wasserman D[4], Cline JM[2], K. Kavanagh K [2]
The Long Term Effects of Ionizing Radiation on Muscle Extracellular Matrix and Perfusion
[1]Cummings School of Veterinary Medicine, Tufts University, North Grafton, MA; [2]Department of Pathology, Wake Forest School of Medicine , Winston Salem, NC; [3]Department of Chemistry, Winston Salem State University, Winston Salem, NC [4]Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN
Abstract accepted; American College of Veterinary Pathologist Annual Meeting 2015
Federal support acknowledged.

5. Abstract

Kavanagh[1], B.R. Pfisterer BR[1], Davis AT[1], Presley TD[2], Williams IM[3], Wasserman D[3], Cline JM[1]
The Long Term Effects of Ionizing Radiation on Muscle Extracellular Matrix and Perfusion In Monkeys
[1]Department of Pathology, Wake Forest School of Medicine, Winston Salem, NC; [2]Department of Chemistry, Winston Salem State University, Winston Salem, NC and [3]Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN
Abstract accepted; Radiation Research Society Annual Meeting 2016
Federal support acknowledged.

6. Abstract

Register TC; Debo R; Michalson K, Lees CJ, Dugan G, Sempowski G, Kavanagh K, Caudell D, and Cline JM. Serum IL-10 and IL-15 Levels are Increased with Prior Exposure to Gamma Irradiation and are Associated with Radiation Sensitive Cardiac Phenotypes.
Abstract accepted for presentation at 2016 Annual Radiation Research Meeting
Federal funding from NIAID and DOD was acknowledged.

Long Term Follow-up of the Late Effects of Acute Radiation Exposure in Primates

Proposal Log Number PR141508

PI: J Mark Cline, DVM, PhD **Org:** Wake Forest Health Sciences **Award Amount:** $9,999,998

Study/Product Aim(s)

To study delayed effects of acute radiation exposure (DEARE) with a focus on our newly-described radiation-induced pathologies, through phenotypic and mechanistic studies, including:

1. Type 2 diabetes mellitus
2. Radiation-induced heart disease (RIHD)
3. Chronic immune impairment with restriction of the antigenic response repertoire; and
4. Radiation-specific genomic signatures underlying 1-3.

Approach

We will use unique nonhuman primate (NHP) resources, including a pre-existing long-term (10+ year) radiation-exposed survivor cohort and development of a prospective cohort allowing baseline pre-exposure assessments and 4 years of follow-up. Complementary immune, metabolic, and interventional studies to be done in mice. Priority will be given to pathways and strategies with high potential for translation to human exposures and outcomes.

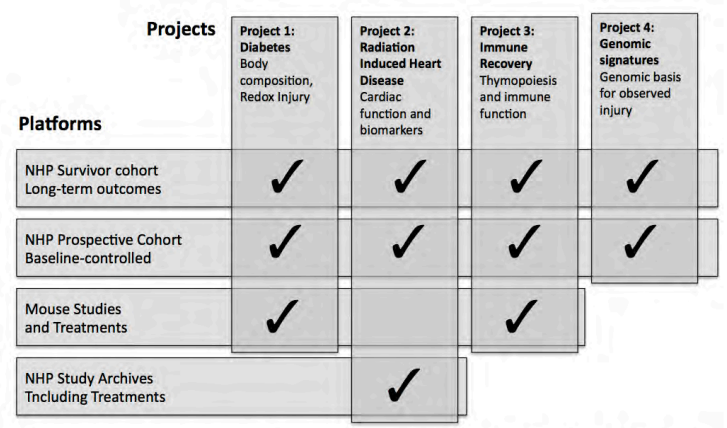


Figure 1. Schematic overview of the matrix relationship between populations of irradiated animals studied and investigative approaches used.

Accomplishments: Organizational and regulatory goals met. Data collection and analysis in progress. Methods development in progress for some novel outcomes, as planned. Initial publications and abstracts, data presented at national meetings.

Timeline and Cost

Activities CY	15	16	17	18	19	20
Oversight/Core Primate Studies						
Proj 1 Diabetes						
Proj 2 Radiation/Heart Disease						
Proj 3 Immune Recovery						
Proj 4 Genomic sequencing and stem cell lines						
Estimated Budget ($K) ***Based on budget period 09/30/15 – 09/29/20. Budget period in ( ) after dollar amounts.***	1	2	3	4	5	
	$2,150,541 (1)	$1,975,657 (2)	$2,030,283 (3)	$1,979,721 (4)	$1,863,796 (5)	

Updated: December 29, 2016

Goals/Milestones

CY15 Goal – Program/Project "Kickoff"
- ☑ Regulatory approvals, establishment of admin structure, scheduling/ planning
- ☑ Animal acquisition and NHP quarantine & sampling/analysis, both cohorts

CY16 Goals – Prospective NHP studies begin, preliminary results presented nationally
- ☑ Exome sequencing and IPSCs from NHP studies (in progress)
- ☑ Irradiation of NHP prospective cohort and post-irradiation sampling/analysis for cardiac, redox, tissue composition, RNA profiling, and biomarker studies; presentation of early results (in progress)
- ☑ Mouse radiation studies: classic model

CY17 Goal – Continued NHP and mouse studies
- ☐ Mouse radiation studies: secondary model
- ☐ Continued analysis of NHP composition/biomarkers as above based on preliminary results; presentation of data

CY18 Goal – Continued NHP and mouse studies
- ☐ Mouse intervention study of HGH and IGF-1 (phenotypic)
- ☐ Continued analysis of NHP composition/biomarkers as above; publication
- ☐ Preliminary cell lineage data from NHPs presented nationally

CY19 Goal – Continued NHP and mouse studies
- ☐ Mouse intervention study of HGH and IGF-1 (functional)
- ☐ Primate studies as above continue; publication

CY20 Goal – Analysis and publication of results
- ☐ Mouse intervention study of HGH and IGF-1 (mechanistic)
- ☐ Necropsy of prospective cohort, analysis of terminal outcomes; publication

Comments/Challenges/ Issues/Concerns
- No changes to timeline at this time
- No changes in quarterly expenditures at this time

Budget Expenditure to Date

Projected Expenditure: $2,150,541
CY 2015/2016

Actual Expenditure: $1,172,887
(Through 9/30/16)

Attachment 2

RADIATION RESEARCH **186**, 55–64 (2016)
0033-7587/16 $15.00

DOI: 10.1667/RR14357.1

Late Effects of Total-Body Gamma Irradiation on Cardiac Structure and Function in Male Rhesus Macaques

Ryne J. DeBo,[1] Cynthia J. Lees, Greg O. Dugan, David L. Caudell, Kris T. Michalson, David B. Hanbury, Kylie Kavanagh, J. Mark Cline and Thomas C. Register[2]

Department of Pathology/Comparative Medicine, Wake Forest University School of Medicine, Winston-Salem, North Carolina

DeBo, R. J., Lees, C. J., Dugan, G. O., Caudell, D. L., Michalson, K. T., Hanbury, D. B., Kavanagh, K., Cline, J. M. and Register, T. C. Late Effects of Total-Body Gamma Irradiation on Cardiac Structure and Function in Male Rhesus Macaques. *Radiat. Res.* 186, 55–64 (2016).

Heart disease is an increasingly recognized, serious late effect of radiation exposure, most notably among breast cancer and Hodgkin's disease survivors, as well as the Hiroshima and Nagasaki atomic bomb survivors. The purpose of this study was to evaluate the late effects of total-body irradiation (TBI) on cardiac morphology, function and selected circulating biomarkers in a well-established nonhuman primate model. For this study we used male rhesus macaques that were exposed to a single total-body dose of ionizing gamma radiation (6.5–8.4 Gy) 5.6–9.7 years earlier at ages ranging from ~3–10 years old and a cohort of nonirradiated controls. Transthoracic echocardiography was performed annually for 3 years on 20 irradiated and 11 control animals. Myocardium was examined grossly and histologically, and myocardial fibrosis/collagen was assessed microscopically and by morphometric analysis of Masson's trichrome-stained sections. Serum/plasma from 27 irradiated and 13 control animals was evaluated for circulating biomarkers of cardiac damage [N-terminal pro B-type natriuretic protein (nt-proBNP) and troponin-I], inflammation (CRP, IL-6, MCP-1, sICAM) and microbial translocation [LPS-binding protein (LBP) and sCD14]. A higher prevalence of histological myocardial fibrosis was observed in the hearts obtained from the irradiated animals (9/14) relative to controls (0/3) ($P = 0.04$, χ^2). Echocardiographically determined left ventricular end diastolic and systolic diameters were significantly smaller in irradiated animals (repeated measures ANOVA, $P < 0.001$ and $P < 0.008$, respectively). Histomorphometric analysis of trichrome-stained sections of heart tissue demonstrated ~14.9 ± 1.4% (mean ± SEM) of myocardial area staining for collagen in irradiated animals compared to 9.1 ± 0.9 % in control animals. Circulating levels of MCP-1 and LBP were significantly higher in irradiated animals ($P < 0.05$). A high incidence of diabetes in the irradiated animals was associated with higher plasma triglyceride and lower HDLc but did not appear to be associated with cardiovascular phenotypes. These results demonstrate that single total-body doses of 6.5–8.4 Gy produced long-term effects including a high incidence of myocardial fibrosis, reduced left ventricular diameter and elevated systemic inflammation. Additional prospective studies are required to define the time course and mechanisms underlying radiation-induced heart disease in this model.

INTRODUCTION

Radiation-induced heart disease (RIHD) is a serious side effect of radiotherapy and/or accidental radiation exposure, characterized by a heterogeneous group of acute and/or chronic physiologic or pathological abnormalities in the heart. Acute conditions include pericarditis, pericardial effusions and arrhythmias. Delayed abnormalities, appearing months to years later, include cardiomyopathy, coronary artery disease, valve defects, conduction abnormalities and myocardial fibrosis. The exact etiology of the acute tissue damage leading to long-term risks is not fully understood and may result from direct and indirect effects. Potential contributors include endothelial injury and dysfunction, DNA damage and altered cardiac gene expression, acute and chronic oxidative stress, microvascular damage, inflammation and other factors (*1–6*). Long-term cardiovascular disease (CVD) incidence data collected from survivors of accidental, malicious and therapeutic exposures highlight the need for well controlled studies to improve our understanding of RIHD pathophysiology so that targeted treatment and prevention strategies can be successfully developed and implemented.

From the Life Span Study research program, which has followed Hiroshima and Nagasaki atomic bomb survivors for more than 50 years, it was found that an estimated excessive relative risk for heart disease was 14% per Gy of exposure. Excess risk was observed even at relatively low doses of radiation (0.5–2 Gy), suggesting the threshold for increased risk to the heart (*7*) is much lower than previously

[1] Radiation Research Society Scholar-in-Training.

[2] Address for correspondence: Department of Pathology, Section on Comparative Medicine, Wake Forest School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157-1040; email: register@wakehealth.edu.

thought. More recently, a dose-dependent increase in RIHD has been observed in residents of contaminated areas after the Chernobyl accident, (*8*), supporting previous findings of increased CVD nationwide in Belarus compared to the pre-Chernobyl disaster era (*9*).

Radiation-induced heart disease is also a late and relatively common serious side effect of radiotherapy and has received increasing attention since it was reported that the heart was at least 10 times more radiosensitive than previously thought (*10*). RIHD is most prevalent among patients treated for breast, chest and thoracic cancer, as well as Hodgkin's disease patients who have received radiotherapy as a part of their medical intervention (*1*, *5*, *11*). CVD after radiotherapy for Hodgkin's disease is the third leading cause of death in Hodgkin's patients (*1*), and the primary cause of non-cancer death among 5-year childhood cancer survivors (*12*). In epidemiological studies such as Surveillance Epidemiology and End Results (SEER) (*13*) and Early Breast Cancer Trialists Collaborative Group (EBCTG) (*14*), it was found that while adjuvant radiotherapy reduced the risk of death from cancer it failed to confer significant survival benefit due to increases in CVD-related morbidity and mortality. Currently, the risk of dying from CVD exceeds the risk of dying from initial or recurrent cancer for many cancer survivors (*15–17*). The relative risk values generally reported in Hodgkin's disease survivors range from 2 to 5 for cardiovascular morbidity (*18–20*), with one study reporting a relative risk of 7.2 (*21*). A recent population-based case-control study in women who received radiotherapy for breast cancer between 1958 and 2001 in Denmark and Sweden showed that the rate of major coronary events increased by 7.4% per Gy of incidental exposure to the heart with no apparent threshold for increased risk (*22*). A meta-analysis of eight randomized trials involving breast cancer survivors showed a 62% increase in cardiac death among patients who were treated with radiation therapy (*23*). Hardenbergh *et al*. (*24*) reported that subclinical cardiac damage occurred in greater than 50% of breast cancer survivors who had received radiation therapy.

Currently, there are no approved treatments or reliable biological markers for RIHD. Traditional markers of cardiac damage or dysfunction do not appear to be useful for the evaluation of radiation-induced cardiac damage (*25*). Therefore, the discovery and development of biomarkers useful in identifying patients likely to experience RIHD would provide the healthcare community with useful tools to target populations for monitoring and therapy. The exact mechanisms underlying RIHD are still largely unclear (*26*); much of what is known has been derived from *in vitro* studies of tissue and cell responses and from *in vivo* studies in small animal models that do not fully approximate human physiology (*19*). Old world monkeys such as the rhesus macaque (*Macaca mulatta*) have close evolutionary ties to humans, making them an invaluable model to study physiologic and pathologic conditions (*27*, *28*). The utility of the rhesus macaque in investigation of radiation countermeasures is well established (*29–32*). In an effort to understand the susceptibility of the macaque to cardiac pathologies similar to those experienced in the human population, and in a long-term effort to identify pathways important in the initiation and progression of RIHD, we investigated the long-term effects of total-body irradiation (TBI) on cardiovascular structure, function and selected circulating biomarkers in adult rhesus macaques.

MATERIALS AND METHODS

Animal Acquisition and Housing

Male rhesus nonhuman primates (NHP) were obtained from the Armed Forces Radiobiology Research Institute (AFRRI), the University of Maryland (UMD) and the University of Illinois at Chicago (UIC). Animals were comprised of a group that had been total-body irradiated with a single dose (6.5–8.4 Gy) approximately 5.6–9.7 years earlier, and a group of age-matched, nonirradiated controls. Animals were screened by intradermal tuberculin testing to exclude tuberculosis, and by immunoassay to exclude simian retroviruses (Washington National Primate Research Center, Seattle, WA). Upon arrival animals were aged based on dentition, weighed, examined by a veterinarian and quarantined for 60 days per standard institutional protocol. All experimental procedures were conducted in compliance with the Wake Forest School of Medicine (WFSM) Institutional Animal Care and Use Committee requirements and followed the Guide for Care and Use of Laboratory Animals.

Basic Clinical Care and Clinical Pathology Assessments

Monkeys were assessed daily for food consumption, defecation, urination and evidence of discomfort or illness. Blood counts and chemistry panels were collected quarterly after quarantine. In the event of low absolute neutrophil counts ($<$250/μl), animals were given parenteral prophylactic antibiotic treatment, consisting of enrofloxacin (10 mg/kg) or ceftiofur sodium (30 mg/kg), until the white blood cell count returned to the normal range and any clinical illness, if present, resolved. Supportive fluid therapy, analgesics (e.g., ketoprofen) and symptomatic care were given as needed based on clinical pathology. Signs of illness were monitored using the methods of Uckun *et al*., as modified from the Children's Cancer Group Clinical Toxicity Criteria (*33*). We have used these procedures with success in previous primate trials of bone marrow-suppressive agents (*34*, *35*). In the event that an animal became moribund or reached grade 4 toxicity by our criteria, they were humanely euthanized following the American Veterinary Medical Associations Guidelines on Euthanasia (*36*).

Diet

Prior to arrival at WFSM (and during the time of irradiation), animals consumed a standard laboratory primate chow diet containing 18% protein, 13% fat and 69% carbohydrates, with a low cholesterol content of 0.023 mg/cal (Monkey Diet 5038; LabDiet®, St. Louis, MO). After arrival at WFSM, animals were transitioned to a moderately atherogenic Western diet developed by WFSM and Purina® (Typical American Diet, TAD; Purina LabDiet as NHP Diet) which contained 18.4% protein, 36.2% fat and 45.4% carbohydrates as percentage of calories provided, and a cholesterol content of 0.18 mg/cal. Animals that became diabetic [hemoglobin A1c (HgbA1c) $>$6.5%] during the study were transitioned back to standard laboratory chow in the interest of health preservation.

Echocardiography

Echocardiography was successfully performed annually on 20 irradiated and 11 control animals using a SonoSite MicroMaxx® Ultrasound System (Bothell, WA). Cardiac morphometric and functional phenotypes included left ventricular diameter at end diastole (LVDd), left ventricular diameter at end systole (LVDs), left ventricular wall fractional shortening percentage (LVDFS%) calculated as [(LVDd – LVDs)/LVDd] × 100 and ejection fraction percentage (EF%). Body surface area (BSA, m^2) was calculated as $BSA = BW^{2/3} \times 0.0969$, utilizing the animal's body weight (kg) obtained at the time of echocardiography to allow normalization of cardiac dimensions to BSA (*37*). Electrocardiography was performed biannually and blood pressure parameters were measured 8–10 times annually.

Lipids and Selected Circulating Biomarkers

Plasma lipids and selected biomarkers were measured longitudinally and/or cross-sectionally in irradiated and control animals. Timing of collections corresponded with the first annual echocardiographic evaluation in this study and had variable postirradiation intervals (~5–8 years, mean time since irradiation = 6 ± 0.14 years). Total plasma cholesterol (TPC), triglycerides (TG) and high-density lipoprotein (HDL) levels were assessed in all subjects as previously described (*38*). Serum/plasma from 27 irradiated and 13 control animals was evaluated for the selected biomarkers listed below. This set included all 20 irradiated and 11 control animals in the echocardiographic evaluation, along with an additional 7 irradiated and 2 control animals that died before the final echocardiographic time point reported in this study. Biomarkers included circulating biomarkers of cardiac damage [N-terminal pro b-type natriuretic protein (nt-proBNP 8-29) (ALPCO® Diagnostics, Salem, NH) and troponin-I (Kamiya Biomedical Company, Seattle, WA), inflammation (soluble intercellular adhesion molecule (s-ICAM) (monkey sICAM-1 Platinum ELISA; eBioscience/Bender MedSystems, Vienna, Austria), C-reactive protein (CRP) (ALPCO® Diagnostics), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) (R&D Systems™, Minneapolis, MN)], microbial translocation (LPS-binding protein (LBP) (Hycult® Biotech, Uden, Netherlands) and soluble cluster of differentiation 14 (s-CD14) (R&D Systems) via commercially available ELISA assays.

Necropsy

Animals that died or were euthanized due to morbidity underwent a complete necropsy for collection and processing of all major systems. Major organs were removed, weighed and tissues were preserved either by snap-freezing in liquid nitrogen, by fixation in 4% paraformaldehyde or by freezing in OCT compound. Gross lesions were photographed at time of necropsy and interpretation of pathologic findings was provided by a board-certified veterinary pathologist (JMC).

Histopathologic Analysis of Cardiovascular and Pulmonary Tissues

Gross evaluation of the thoracic and abdominal aorta, iliac arteries, carotid arteries, coronary arteries, cerebral arteries, left ventricle (LV), right ventricle (RV) and interventricular septum (IVS) was performed. Masson's trichrome was used to stain sections of LV, RV and IVS for estimation of fibrosis. Stained sections were digitally imaged using a Hamamatsu NanoZoomer (Hamamatsu, Japan). Histomorphometric analysis of these digitized slides was performed using ImagePro Premier® (Media Cybermetics Inc., Rockville, MD). Whole sections were evaluated using computer-assisted digitization defining representative colors of stained collagen (blues) to determine the collagen stain positive fraction. Briefly, the region of interest was manually traced to determine the area in $pixels^2$. The image was manually color thresholded to select pixels meeting ''blue'' staining criteria, which were then automatically counted to obtain the stained area of the section in $pixels^2$. Collagen area fraction was then determined using the following formula: percentage area collagen positive = (blue area/total area) × 100.

TABLE 1
Frequency Distribution of Cardiac Fibrosis in Irradiated and Nonirradiated Rhesus Macaques

	No fibrosis	Fibrosis	Total
Control	3	0	3
Irradiated	5	9	14
Total	8	9	17
Odds ratio	-	-	12
P value	-	-	**0.04**[a]

[a] Significance ($P < 0.05$) indicated in bold face.

Statistics

The effect of radiation exposure on incidence rates was tested using a chi-square analysis. Physical, echocardiographic and blood pressure phenotypes were analyzed via analysis of variance (ANOVA) or repeated measures ANOVA (RMANOVA). The effect of radiation exposure on these parameters at individual time points was evaluated post hoc using Bonferroni correction. Circulating markers and plasma lipids were analyzed using independent two-tailed *t* tests. In the event that two groups had unequal standard deviations, a Welch's *t* test correction was used. Data are expressed as means ± standard error of the mean (SEM). The above statistical tests were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Pearson's correlation coefficients were calculated to assess relationships between biomarkers and echocardiographic and blood pressure phenotypes across and within the control and irradiated groups. All pairwise correlation analyses were performed using Statistica version 12 (StatSoft Inc., Carlsbad, CA). $P < 0.05$ was considered significant.

RESULTS

Cardiac Structural and Functional Phenotypes

Fourteen irradiated and 3 control animals were euthanized during the study and their tissues were available for analysis at the time of this report. Elapsed time between irradiation and death was 5.6 ± 0.59 years (range 2.2–9.25). Figure 1A and B are photomicrographs of trichrome-stained sections of the LV, illustrating loss of normal cardiomyocyte architecture and replacement with collagen and fat in the irradiated animal. Compared to the myocardium of control animals, irradiated myocardium often exhibited separation of cardiac myofibers by intervening fibrous connective tissue, loss of normal architecture, with deranged cellular orientation and enlargement of cardiac myocytes (Fig. 1C–F). Gross and qualitative microscopic histological analysis of the 17 hearts available from euthanized animals revealed a significantly higher incidence of myocardial fibrosis in 9 of 14 irradiated animals compared to 0 of 3 control animals ($P = 0.04$, χ^2) (Table 1). Histomorphometric analysis of trichrome-stained sections of myocardium from these animals revealed a mean of 14.9% (±1.35) percentage collagen in irradiated compared to 9.1% (±0.86) in control animals ($P = 0.17$, *t* test).

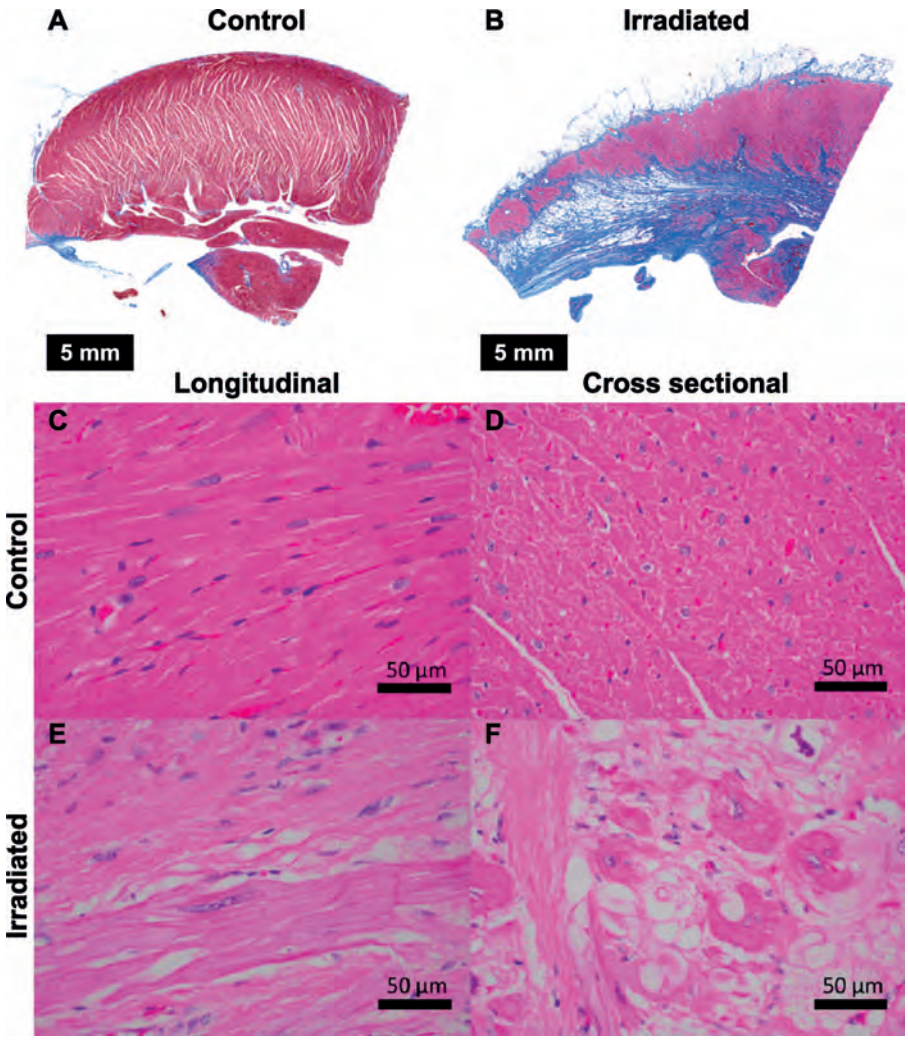


FIG. 1. Effects of prior exposure on indices of cardiac fibrosis: Panels A and B: Trichrome-stained sections of myocardium from the left ventricle of a control animal (panel A) and of a 6.5 Gy irradiated animal 2.5 years earlier (panel B) (scale bars = 5 mm). Panels C–F: Longitudinal and cross-sectional H&E stained myocardium. Panels C and D: control myocardium. Panels E and F: Irradiated myocardium exhibiting marked separation of cardiac myofibers by intervening fibrous connective tissue, loss of normal architecture, cellular orientation and enlargement of cardiac myocytes. Photomicrographs were taken at 40×. Scale bar for panels C–F = 50 µm.

Echocardiographic Phenotypes

Selected outcomes for echocardiographic phenotypes are shown in Fig. 2. Thirty-one animals were evaluated annually for 3 consecutive years using echocardiography. The estimated mean age at the final assessment was 11.2 (± 0.3) years for the 11 control animals, compared to 11.8 (± 0.5) years for the 20 irradiated animals. At the final echocardiographic assessment monkeys had an average postirradiation interval of 8.6 ± 0.2 years and had been consuming the Western diet for an average of 3.7 ± 0.1 years. Echocardiographic LVDd and LVDs were significantly smaller in irradiated animals compared to controls (LVDd: $P = 0.001$ and LVDs: $P = 0.008$, RMANOVA; Fig. 2A and B). Correction for body surface area (BSA) (*39*) did not significantly alter outcomes (LVDd: $P = 0.007$ and LVDs: $P = 0.017$, RMANOVA, data not shown). Irradiated animals tended to have greater LVDFS% ($P = 0.054$, RMANOVA) (Fig. 2C). Radiation had no effect on EF% ($P = 0.930$, RMANOVA; data not shown). Irradiated animals tended to have higher mean annual heart rate ($P = 0.06$, RMANOVA), but were not different from controls in systolic, diastolic or mean arterial blood pressure (all $P > 0.35$, Table 2).

Biomarkers

Cardiac. Circulating troponin-I and nt-proBNP fragment (8–29) were below the detection limits of the ELISA assays, which were 0.156 ng/ml for cardiac troponin-I and 171 pmol/l for nt-proBNP.

Inflammation. Significantly higher levels of circulating MCP-1 were observed in the irradiated animals ($P = 0.016$) (Fig. 3A). Irradiated animals showed a trend towards

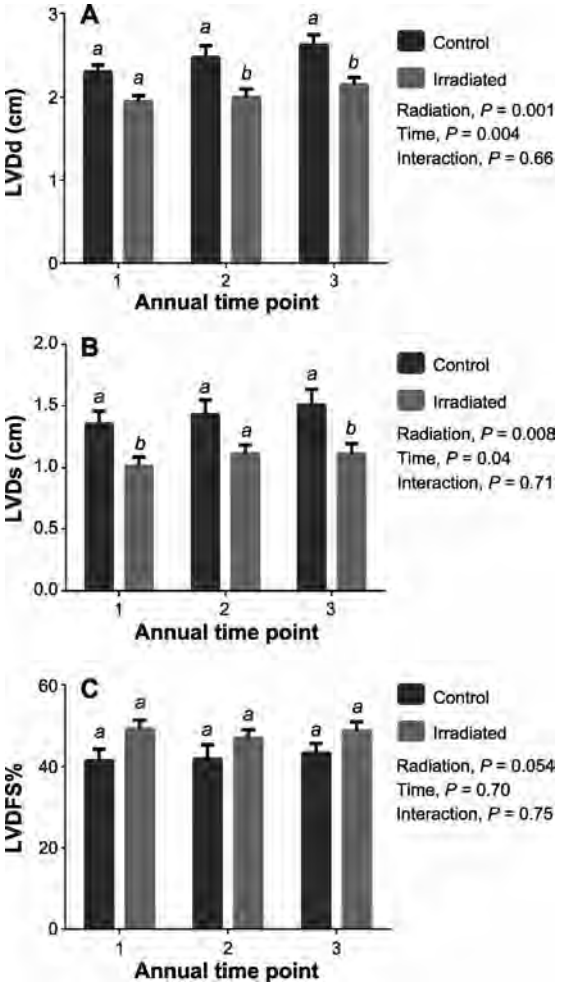


FIG. 2. Effects of prior exposure on cardiac function via echocardiographic assessments. Panels A and B: Echo left ventricular diastolic and systolic diameters (LVDd and LVDs, respectively) were significantly smaller in irradiated animals (RMANOVA, $P = 0.001$ and $P = 0.008$). Panel C: Fractional shortening percentage (LVDFS%) tended to be greater in irradiated animals (RMANOVA, $P = 0.054$). Data are mean ± SEM. Control (n = 11); irradiated (n = 20) across all years. Unlike letters denote significance between control and irradiated animals within time points with P values < 0.05.

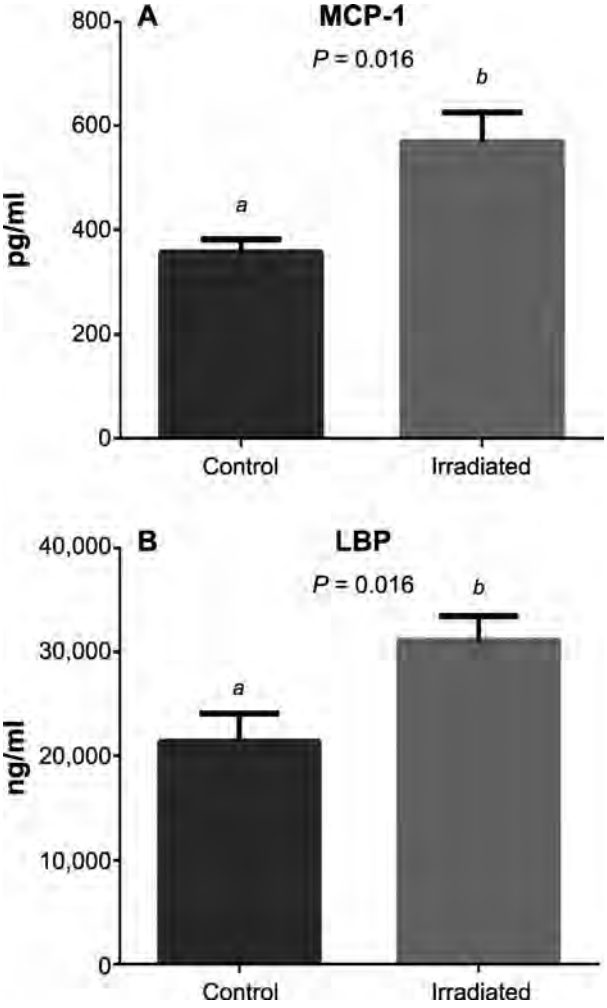


FIG. 3. Effects of prior exposure on circulating MCP-1 and LBP. Monocyte chemoattractant protein-1 (MCP-1; panel A) and LPS-binding protein (LBP; panel B) were significantly higher in irradiated animals (t test, $P = 0.016$). Data are mean ± SEM. Unlike letters denote significance between control and irradiated animals with P values < 0.05.

TABLE 2
Effects of Prior Irradiation on Heart Rate and Blood Pressure in the Echocardiographic Subset of Animals

	Time point 1		Time point 2		Time point 3		P value		
	Control	TBI	Control	TBI	Control	TBI	Time	TBI	Time × TBI
n =	11	21	11	21	11	21			
Heart rate (beats per min)	138.7	151	128	137	125	132	**0.0001**[a]	0.06	0.31
SEM	4.4	3.1	4.6	2.8	4.4	2.8			
Systolic BP (mm Hg)	128	127	126	123	126	127	0.067	0.83	0.27
SEM	2.9	2.8	2.9	2.6	2.9	3.1			
Diastolic BP (mm Hg)	68.2	73.3	67.4	69.9	70.8	72.4	0.10	0.36	0.42
SEM	1.8	2.3	3.3	2.0	2.4	2.5			
Mean BP (mm Hg)	91.6	94.4	88.6	90.8	91.6	92.3	0.06	0.57	0.73
SEM	2.3	2.4	3.2	2.1	1.9	2.5			

Notes. Time points 1, 2 and 3 correspond to years 1, 2 and 3. Data were evaluated by repeated measures ANOVA. SEM = standard error of the mean. BP = blood pressure.

[a] Significance ($P < 0.05$) indicated in bold face.

TABLE 3
Effects of Prior Radiation Exposure on Circulating Levels of hsCRP, IL-6, sCD-14 and sICAM-1

	Control (n = 13)	TBI (n = 27)	*P* value
hsCRP (ng/ml)	986	1,507	0.12
SEM	227	198	
IL-6 (pg/ml)	1.95	4.25	0.12
SEM	0.46	0.96	
sCD-14 (ng/ml)	1,150	1,286	0.25
SEM	77	71	
sICAM (U/ml)	24.4	30.6	0.53
SEM	3.0	6.7	

Note. Data are mean ± standard error of mean (SEM).

elevated levels of circulating CRP and IL-6 ($P = 0.12$), and there was no effect of radiation on s-ICAM (Table 3).

Microbial translocation. Significantly higher levels of circulating LBP were observed in the irradiated animals ($P = 0.016$) (Fig. 3B). Previous exposures to radiation had no effect on circulating levels of sCD14 at the time point evaluated in this study (Table 3).

Relationships between biomarkers and other phenotypes. Circulating levels of MCP-1 were positively correlated with circulating levels of LBP ($r = 0.37$, $P = 0.018$, $n = 40$). MCP-1 and LBP were not correlated with cardiac functional phenotypes (all $P > 0.05$, data not shown).

Diabetes, Body Weight and Plasma Lipids

Across the entire cohort of 59 subjects, a high incidence of type 2 diabetes mellitus (T2DM, defined as HgbA1c > 6.5%) was observed in the irradiated animals (13 of 46), while there was no T2DM in the control animals (0 of 13) ($P = 0.03$, χ^2). Diabetic animals had significantly higher HbA1c values than controls ($P = 0.0001$) and irradiated nondiabetic monkeys ($P = 0.0001$), while controls were not different from irradiated nondiabetic monkeys ($P = 0.9$) (Table 4). Body weight and plasma lipids were influenced by exposure and diabetes status. Irradiated nondiabetic animals had significantly lower body weights than control animals and irradiated diabetic animals (all $P < 0.05$). TBI animals with diabetes exhibited higher TG than controls ($P < 0.005$) and TBI nondiabetic monkeys ($P = 0.003$), and had lower HDLc compared to control ($P < 0.03$) and nondiabetic irradiated monkeys ($P < 0.002$). Further details on the pathophysiology of T2DM in irradiated macaques have been previously published elsewhere (*40*).

Effects of Diabetes on Individual Phenotypes

Cardiac phenotypes (ventricular diameters, heart rate and blood pressure) and nonlipid biomarkers did not differ between diabetic and nondiabetic irradiated animals (data not shown), and HgbA1c concentrations were not correlated with cardiac phenotypes (all $P > 0.05$), but were associated with body weight in animals in the echocardiographic subset (all $P < 0.01$).

DISCUSSION

Our data indicate that male rhesus macaques receiving total-body ionizing radiation (6.5–8.4 Gy) 5.6–9.7 years earlier exhibited the following: 1. Increased incidence of gross myocardial fibrosis at time of death ($P < 0.05$); 2. Smaller cardiac dimensions ($P < 0.01$); 3. Increased incidence of T2DM ($P = 0.03$); and 4. Higher levels of circulating MCP-1 and LBP than control animals. With the exception of a tendency for higher heart rates in irradiated animals, no significant differences were observed in standard clinical measures of cardiac health and function, such as systolic or diastolic blood pressure, with the caveat that these were conducted while animals were under anesthesia.

TABLE 4
Effects of Prior Radiation Exposure and Diabetes on Plasma Lipids in the Echocardiographic Subset of Animals: Time Point 2 Only

					P values		
	Control (n = 11)	TBI, no T2DM (n = 13)	TBI + T2DM (n = 8)	ANOVA *P* value	Control vs. TBI, no T2DM	Control vs. TBI + T2DM	TBI, no T2DM vs. TBI + T2DM
HbA1c (%)	4.5	4.7	9.6	**0.0001**[a]	0.9	**0.0001**[a]	**0.0001**[a]
SEM	0.1	0.1	0.6				
Body weight (kg)	12.9	8.8.	14.2	**0.0001**[a]	**0.0004**[a]	0.45	**0.0001**[a]
SEM	1.0	0.4	0.7				
TPC (mg/dl)	164	187	213	0.19	0.57	0.16	0.57
SEM	18	9	30				
TG (mg/dl)	88.9	76.5	484	**0.002**[a]	0.99	**0.005**[a]	**0.003**[a]
SEM	9.1	3.5	179				
HDL (mg/dl)	71.6	80.6	47.8	**0.003**[a]	0.49	**0.03**[a]	**0.002**[a]
SEM	7.6	4.1	5.9				

Notes. All lipid measurements were performed at a single time point. All lipids were evaluated using an unpaired two-tailed *t* test and ANOVA to evaluate the effect of T2DM on lipid measurements. Between group ANOVA *P* values. Post hoc analyses corrected for multiple comparisons via Tukey's HSD. TBI = total-body irradiation; T2DM = type 2 diabetes mellitus; SEM = standard error of the mean.

[a] Significance ($P < 0.05$) indicated in bold face.

Gross histopathological evaluation of postmortem myocardium revealed a significant increase in incidence of myocardial fibrosis at time of death in previously irradiated animals. Image analysis of trichrome-stained sections of myocardium suggested a tendency for increased collagen deposition in irradiated animals. These results suggest that TBI in the 6.5 Gy and above range predisposed animals to the development of myocardial fibrosis as a late effect and expand on previously published data from Wondergem *et al*. (*39*) using a similar model.

Irradiated animals exhibited significantly smaller LVDd and LVDs diameters than controls before and after adjustment for BSA. Wondergem *et al*. (*39*) also observed smaller LVDd and LVDs in irradiated male and female rhesus macaques ($P = 0.0002$ and 0.038, respectively), although correction for BSA in their study reduced significance for LVDs ($P = 0.07$). This modest difference between studies may be in part due to the inclusion of females and postirradiation treatment with bone marrow transplants (*39*), which were not used in our study (*41–43*). Similar observations were recently reported in a murine model of radiation exposure, where LVDs was significantly lower ($P < 0.05$) and LVDd tended to be lower ($P = 0.06$) (*44*). The smaller LV dimension measurements are consistent with a restrictive cardiomyopathy due to fibrosis, although reduced relaxation or blunted growth of the heart postirradiation could play a role.

Functional parameters suggest that systolic function (LV fractional shortening) was preserved in the irradiated cohort, as irradiated animals tended to have higher LVDFS% ($P = 0.06$). In the murine model noted above, fractional shortening also tended to be increased ($P = 0.10$) (*44*). The mechanisms underlying the tendency towards increased fractional shortening are unclear, but may represent a compensatory response to promote adequate cardiac output and blood flow.

In our previous work with female cynomolgus monkeys, we showed that rapid dose-dependent increases in circulating cytokines including MCP-1 soon after irradiation (*42*). MCP-1 is an important mediator of inflammation and monocyte recruitment. In humans, increased serum levels of MCP-1 are associated with several cardiovascular conditions including subclinical atherosclerosis, acute coronary syndromes, congestive heart failure and peripheral arterial disease (*45–48*). In a previous study unrelated to radiation effects, we found that MCP-1 was significantly correlated with atherosclerosis in coronary, carotid and iliac arteries of ovariectomized female cynomolgus macaques consuming an atherogenic Western diet (*49*). The current finding that MCP-1 and LBP were elevated several years postirradiation (5–8 years) is suggestive of a persistent pro-inflammatory state, which may have a long-lasting impact on many tissues, including the heart. Increased incidence of T2DM and higher circulating MCP-1 levels were also observed in a subset of the animals in the current study, although these phenotypes did not appear to be correlated with one another or causally linked (*40*).

Significant elevations in LBP, a marker of microbial translocation and mediator of the innate immune response to microbial infection, may be indicative of chronic LPS exposure (*50*) due to damage to the gastrointestinal system, which is particularly radiosensitive and can become transiently or permanently leaky as a result of radiation exposure (*51*). LBP is an acute phase protein which recognizes bacterial LPS and complexes with sCD14, initiating production of pro-inflammatory interleukins and cytokines via Toll-like receptor (TLR) interactions (*52*, *53*), and enhancing macrophage activation during acute injury processes (*54*). Recently, LBP has gained attention as a potential marker of coronary artery disease/atherosclerosis (*55*, *56*) and cardiovascular-related mortality (*57*).

Circulating troponin-I and nt-proBNP data presented here were obtained using standard ELISA assays, which lack sensitivity to detect low, subclinical levels of these biomarkers. Previous studies of these markers in humans undergoing radiotherapy have produced mixed results. In a study of 30 patients undergoing thoracic radiotherapy with chemotherapy, no significant elevations in troponin, nt-proBNP or creatine kinase-myocardial band (CK-MB) were observed after radiotherapy (*58*). However, recent studies suggest BNP may be useful as an acute marker of early radiotherapy-related cardiovascular impairment in left-sided breast cancer patients (*59*), and in patients receiving thoracic radiotherapy with high-dose heart exposure (*59*, *60*). Nevertheless, the majority of patients receiving greater than 20 Gy to the heart during thoracic radiotherapy did not exhibit increased levels of troponin I (*60*). The follow-up times in the above studies were shorter than those in the current study.

Inflammation and diabetes are well-established factors in the development and progression of CVDs (*61*). While the incidence of T2DM and the levels of inflammatory MCP-1 and LBP were increased in the irradiated cohort, we did not find evidence that either T2DM or these inflammation-associated markers were specifically associated with the cardiac structural or functional parameters in the study (data not shown). Ongoing evaluations of molecular and cellular phenotypes in the heart and coronary arteries of these and other subjects should provide insights into the determinants of atherosclerosis and cardiac damage.

Due to the opportunistic nature of this study, certain limitations were inherent and unavoidable. The irradiated animals used in this study were survivors of potentially lethal TBI, and perhaps possessed unique protective genotypes and/or phenotypes not representative of all rhesus macaques. The pro-inflammatory effects of TBI observed during this study may differ from that of localized therapy, as it represents a much broader exposure of multiple tissue types including the heart. Elevated circulating biomarkers may originate from

numerous tissues within the animal, and information is lacking as to the specific tissue origin of the elevated MCP-1, although LBP is presumed to be derived from the liver. Nevertheless, off-target effects of region-specific radiation treatment, either through scatter, pro-inflammatory and/or oxidative factors generated locally and distributed through the circulation is known to occur. The experimental design for this group of animals is not prospective in the sense that we have no baseline data or samples available prior to the time of exposure, similar to situations involving survivors of a radiation accident. Additionally, our animal cohort was consuming a standard low-fat chow diet at the time of irradiation, and was not transitioned to a Western diet until after arriving at our institution. The Western diet was utilized to better represent physiologic conditions present in a large proportion of Western society. Unfortunately, this design does not allow for an assessment of the effects of diet on radiosensitivity. Lastly, while the Western diet may have contributed to the development of diabetes in the irradiated animals, it is not possible to fully explore the interactions between diet, diabetes and cardiac or other phenotypes since the animals are generally placed on chow once diabetes becomes evident.

In summary, TBI given as single doses of 6.5–8.4 Gy produced long-lasting myocardial injury, altered heart structure and function and elevated levels of MCP-1 and LBP consistent with a pro-inflammatory state. Additional studies in the NHP model are required to more fully understand the sequence of events postirradiation, which can promote RIHD, in order to identify useful biomarkers and develop strategies for mitigating the process.

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Attachment 3 - Radiation Research Society Abstract 2016

Multisystemic late effects of radiation exposure in nonhuman primates

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Introduction: The major burden of radiation injury lies in delayed effects. These long-term effects of acute radiation exposure include degenerative cellular changes leading to organ failure; fibrosis; and neoplasia.
Procedures: We present here the long-term adverse effects of single-dose whole-body exposure at 3.5 to 8.4 Gy in over 100 rhesus monkeys exposed at a median age of 4 years and observed for up to 10 years, in comparison to 16 non-irradiated controls. Some animals were exposed prior to puberty (<3.5 years of age), and the remainder as young adults (up to 10 years of age). Observations included clinical examinations, imaging (whole body CT and brain MRI), cognitive testing, hematology, clinical chemistry, and for those animals dying under observation, necropsy and histopathology.
Results: Major disease processes identified to date include (1) type II diabetes mellitus with islet hyperplasia, amyloidosis, and increased peripheral insulin resistance; (2) myocardial fibrosis and reduced left ventricular diameter; (3) immune compromise manifested as impaired vaccine responses, skin and wound infections, bronchopneumonia, pericarditis, and sepsis; (4) chronic pulmonary disease including pneumonitis, fibrosis, and epithelial dysplasia; (5) neoplasms including hematopoietic, epithelial, mesenchymal and neuroendocrine types; (6) focal MRI-detected brain lesions; (7) renal impairment, and (8) elevated circulating markers of inflammation. Other stereotypical radiation effects (gonadal atrophy, cataracts) were predictably seen. Multiple disorders in the same animal were common, with diabetes being the most common co-morbid condition.
Conclusions: Our growing body of data indicates that a substantial burden of disease is present in long-term survivor non-human primates, including complex patterns of co-morbidity; metabolic and cardiac disease; injury of “high-dose” tissues such as brain at lower doses than anticipated; immunosuppression and inflammation; and pathologies including both loss/fibrous replacement of functional tissue and cytoproliferative disorders including neoplasms. Typically more than one disease is present in a given animal, necessitating an integrative approach.
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Attachment 4 - Radiation Research Society Abstract 2016

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THE LONG TERM EFFECTS OF IONIZING RADIATION ON MUSCLE EXTRACELLULAR MATRIX AND PERFUSION
Abstract accepted; American College of Veterinary Pathologist Annual Meeting 2015
Federal support acknowledged.

Abstract: A newly recognized delayed effect of radiation exposure in human and nonhuman primates is type 2 diabetes mellitus (T2DM), but the mechanisms are unknown. Our prior monkey studies suggest skeletal musculature is a primary site contributing to radiation-induced insulin resistance. We aimed to determine differences in skeletal muscle extracellular matrix (ECM) and perfusion in rhesus monkeys (*Macaca mulatta*) exposed to whole body irradiation (6.5-8.4Gy) 6-10 years earlier. Skeletal muscle biopsies and plasma samples from rhesus monkeys were collected; non-irradiated control monkeys, irradiated non diabetic monkeys, and irradiated T2DM monkeys (n=8/group). Muscle transforming growth factor beta (TGFβ), vascular endothelial growth factor (VEGF), and heat shock protein (HSP) 90 concentrations were determined. Collagen 1, 2, and 4 abundance was measured as % area by immunohistochemistry. Plasma nitrite and nitrate (NO_2 and NO_3) concentrations were measured as biomarkers of endothelial nitric oxide synthase activity. ECM remodeling in skeletal muscle was evident post-irradiation with the collagen 1:4 ratio being significantly less in irradiated monkeys($p=0.02$) indicating relative overproduction of Col 4 is a persistent effect of radiation. T2DM monkeys had the greatest collagen 4 deposition ($p=0.04$). A consistent finding was higher TGFβ levels in irradiated monkeys ($p=0.10$). HSP90 levels were 32% less in irradiated animals ($p=0.0004$) and also was further suppressed by diabetes ($p=0.03$). Only T2DM monkeys had lower plasma NO_3 concentrations ($p=0.04$), and NO_3 was significantly associated with VEGF levels in muscle. ECM remodeling endpoints, Col 4 and TGFβ, were significantly associated with greater triglyceride and less VEGF in muscle. We demonstrate that even many years after radiation exposure, there is ongoing ECM remodeling in muscle that may lead to lower perfusion and greater intramuscular lipid deposition. These changes are exaggerated in T2DM. Strategies to limit ECM remodeling post-irradiation may preserve metabolic function with restoration of normal insulin-capillary perfusion and subsequent T2DM risk.

Attachment 5 - Radiation Research Society Abstract 2016

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THE LONG TERM EFFECTS OF IONIZING RADIATION ON MUSCLE EXTRACELLULAR MATRIX AND PERFUSION IN MONKEYS
Abstract accepted; Radiation Research Society Annual Meeting 2016
Federal support acknowledged.

A newly recognized delayed effect of radiation exposure in human and nonhuman primates is type 2 diabetes mellitus (T2DM), but the mechanisms are unknown. Our prior monkey studies suggest skeletal musculature is a primary site contributing to radiation-induced insulin resistance. We aimed to determine differences in skeletal muscle extracellular matrix (ECM) and perfusion in rhesus monkeys (*Macaca mulatta*) exposed to whole body irradiation (6.5-8.4Gy) 6-10 years earlier. Skeletal muscle biopsies and plasma samples from rhesus monkeys were collected; non-irradiated control monkeys, irradiated non diabetic monkeys, and irradiated T2DM monkeys (n=8/group). Muscle transforming growth factor beta (TGFβ), vascular endothelial growth factor (VEGF), and heat shock protein (HSP) 90 concentrations were determined. Collagen 1, 2, and 4 abundance was measured as % area by immunohistochemistry. Plasma nitrite and nitrate (NO_2 and NO_3) concentrations were measured as biomarkers of endothelial nitric oxide synthase activity. ECM remodeling in skeletal muscle was evident post-irradiation with the collagen 1:4 ratio being significantly less in irradiated monkeys($p=0.02$) indicating relative overproduction of Col 4 is a persistent effect of radiation. T2DM monkeys had the greatest collagen 4 deposition ($p=0.04$). A consistent finding was higher TGFβ levels in irradiated monkeys ($p=0.10$). HSP90 levels were 32% less in irradiated animals ($p=0.0004$) and also was further suppressed by diabetes ($p=0.03$). Only T2DM monkeys had lower plasma NO_3 concentrations ($p=0.04$), and NO_3 was significantly associated with VEGF levels in muscle. ECM remodeling endpoints, Col 4 and TGFβ, were significantly associated with greater triglyceride and less VEGF in muscle. We demonstrate that even many years after radiation exposure, there is ongoing ECM remodeling in muscle that may lead to lower perfusion and greater intramuscular lipid deposition. These changes are exaggerated in T2DM. Strategies to limit ECM remodeling post-irradiation may preserve metabolic function with restoration of normal insulin-capillary perfusion and subsequent T2DM risk.

Attachment 6 - Radiation Research Society Abstract 2016

Serum Interleukin-10 and Interleukin-15 levels are increased with prior total body gamma irradiation exposure and associated with radiation sensitive cardiac phenotypes

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Abstract:
We recently reported long term / delayed effects of prior acute total body irradiation in a nonhuman primate model of radiation exposure, which included increases in incidence of diabetes (Kavanagh et al., Rad Res 2015) and radiation induced heart disease and cardiac fibrosis (DeBo et al., Rad Res 2016). In the present study we examined the long term effects of prior TBI exposure on circulating cytokine profiles in expanded cohorts of male rhesus macaques. Monkeys had received single whole body doses of 3.5 to 8.5 Gy up to 10 years prior to blood sampling. Serum samples were assessed using multiplex biomarker profiling.
Initial findings suggest that previously irradiated monkeys (n=70) had significantly elevated serum levels of interleukin-10 (IL-10) and interleukin-15 (IL-15) (both $p<0.03$) relative to a companion cohort of non-irradiated male rhesus monkeys (n=14). Within irradiated animals, serum levels of IL-10 and IL-15 were significantly inversely associated with echocardiographically determined diameters of the left ventricle of the heart, which were previously shown to be significantly smaller in irradiated animals (DeBo et al., Rad Res 2016). In addition, IL-15 was inversely associated with left ventricular posterior wall thickness ($p<0.05$) and ejection fraction ($p<0.05$). Among irradiated animals, serum IL-10 and IL-15 levels were similar among diabetic and non-diabetic monkeys and were not correlated with hemoglobin A1c levels. The results suggest long lasting effects of TBI on circulating IL-10 and IL-15, both of which were associated with radiation sensitive cardiac phenotypes. These findings may provide new avenues of investigation for elucidation of mechanisms underlying long-term delayed effects of acute radiation exposure on the body and on the heart.
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